EXPRESSION AND FUNCTION OF LEUKEMIA INHIBITORY FACTOR AND INTERLEUKIN-6 GENES DURING PORCINE PREGNANCY

BY

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by

Tomislav Modrić

To my dearest wife Sanja, for all the encouragement and help.

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The hematopoietic cytokines, leukemia inhibitory factor (LIF) and interleukin-6 (IL-6), are signaling molecules present in the uterine luminal fluid of many species, which are developmentally synthesized and secreted by maternal and embryonic tissues. LIF is essential for implantation in the mouse, as well as for differentiation of the embryonic inner cell mass. LIF and IL-6 modulate the biosynthesis of various proteinases and their specific inhibitors. Antileukoproteinase (ALP) is a proteinase inhibitor coexpressed with hematopoietic cytokines in porcine endometrium and placenta. It is expressed most abundantly in uterine endometrium of species with epitheliochorial placentation, as opposed to the species exhibiting endotheliochorial or hemochorial placentation. Thus, ALP has been proposed to protect endometrium from erosion by conceptus-elicited proteinases. We examined by RT-PCR the temporal expression of LIF and IL-6 cytokine genes as well as that encoding the cytokine-binding subunit of the LIF receptor (LIFR-β) in the porcine female reproductive tract during pregnancy. The highest amounts of LIF, LIFR-β and IL-6 mRNAs were found in endometria and placentae during the early post-implantation period, suggesting a potential role for these cytokines in feto-maternal developmental events. IL-6 mRNA was expressed in the Jag-1, cell line derived from day

14 porcine trophoblast, suggesting a potential role for IL-6 in implantation of pig blastocysts. LIF mRNA was most abundantly expressed in day 30 placentae, whereas LIFR-β mRNA abundance was greatest in the corresponding endometrium, suggesting the involvement of LIF as a signaling molecule at the feto-maternal interface. We examined by Northern dot-blot analysis the *in vitro* effects of hrLIF and hrIL-6 on the expression of ALP mRNA in pregnant porcine endometrium. The treatments of day 12 endometrial explants with hrLIF, as well as with hrIL-6, resulted in increased ALP expression. The stimulatory effects of LIF and IL-6 indicate that a post-implantation role for these cytokines may be to determine the degree of endometrial erosion by the conceptus, as mediated by the cytokine-induced proteinase inhibitors. These findings indicate that LIF and IL-6 may have a role in feto-maternal signaling, such as regulation of placentation.

CHAPTER 1
INTRODUCTION

Blastocyst implantation is a critical event in porcine reproduction, during which time 30% of conceptuses do not survive. This process is orchestrated by many molecular signals, released from the ovaries, conceptuses and uterus. The main implantation signal, released by the periimplantation porcine conceptus, is estrogen, with the peak of secretion between days 11 and 12, *post estrus* (p. e.). During pregnancy, many endometrial proteins are developmentally expressed and some of these have demonstrated roles in orchestration of physiological processes required for pregnancy and development. During the periimplantation period (days 11-18, p. e.), insulin-like growth factor-I and -II are present in uterine luminal fluids (ULF). Both of these factors are thought to influence the expression of steroidogenic enzyme genes, such as those encoding cytochromes $P450_{C17}$ and $P450_{arom}$, in porcine embryos. The cytokines IL-6, LIF, interferon-gamma (IFN-γ), and granulocyte-macrophage colony stimulating factor (GM-CSF) are also present in porcine ULF at the time of implantation. In other species, some of these cytokines are presumed to mediate various aspects of embryo implantation or growth, with LIF being an essential factor for implantation of mouse blastocysts. However, different species have evolved different reproductive strategies, and what is essential for one species may not be critical or even necessary for others. The expression patterns of cytokine genes are best known for the mouse and human, while research of domestic animal species lags behind in this area. As a consequence, very little is known about the developmental expression of hematopoietic and other cytokines in porcine reproductive tract and conceptuses. Cytokines are secreted by various tissues and cell types. Knowing that cytokines from one cell type may induce synthesis of other cytokine(s) in the same or different cell types, one can expect complex interactions between different cell types to be mediated by cytokines.

There is growing interest among reproductive physiologists in tissue remodeling processes, since the endometrium and conceptuses undergo many developmental changes due to tissue remodeling. The most striking morphological change in porcine conceptuses, that is probably the result of tissue remodeling, is elongation, which takes place just before implantation. The uterus also undergoes histological and functional changes in order to accommodate growing conceptuses, and these changes are a result of tissue remodeling, as well. Enzymes regulating the degree and type of tissue remodeling are proteinases and proteinase inhibitors. LIF and IL-6 have been found to stimulate or inhibit many genes encoding proteinases or proteinase inhibitors, in general stimulating the synthesis of proteinase inhibitors, while inhibiting the synthesis of proteinases. Therefore, hematopoietic cytokines, if expressed during porcine pregnancy, may regulate tissue remodeling of either endometrium or conceptuses, or both. Obtaining more information on the developmental expression pattern of hematopoietic cytokines during pregnancy in swine will allow us to speculate about their possible roles in pregnancy of swine and other ungulates, all of which exhibit the epitheliochorial type of placentation.

The present studies were conducted to: 1) investigate the expression pattern of the LIF, LIFR-β, IL-6 and β_2m genes in pig endometria and conceptuses during early pregnancy; 2) clone the porcine LIFR-β complementary DNA (cDNA) fragment and compare it to sequences of the same cDNAs from different species; and 3) investigate effects of LIF and IL-6 on the expression of potential downstream endometrial genes normally expressed during pregnancy, namely ALP and β_2m.

Data obtained from these studies may help us better understand the cytokine signaling pathways that mediate interactions of the pig blastocyst and uterus. In the long term, such knowledge may help us to devise strategies to reduce embryonic mortality, and thereby, improve reproductive efficiency in swine.

CHAPTER 2
LITERATURE REVIEW

Reproduction in Swine

Estrous Cycle and Pregnancy

As in other aspects of reproductive strategies, pregnancy is an event wherein diversity among mammalian species is evident. The domestic pig is a polyestrous, non-seasonal animal that reaches puberty at 4-9 months of age. Its average estrous cycle lasts 21 days, and the period of estrus lasts 2-3 days. Ovulation takes place about 46 hours after the onset of estrus. Pregnancy in swine lasts between 112 and 115 days. The litter size is 8-10 piglets for a gilt and 10-16 for a sow (Fraser et al., 1986). Compared to other species, the pig has a relatively long period during which the conceptus is unattached to the uterine endometrium. Porcine conceptuses remain unattached for 12 days, while for example, mouse blastocysts attach to the uterine endometrium at 4-6 days (Abrahamsohn and Zorn, 1993) and in the human at 6-7 days, post-fertilization (Hafez, 1979).

Embryonic Development

The porcine ovum becomes fertilized in the oviduct, on days 1-2, p. e. It undergoes its first cleavage on day 2, p. e. (Patten, 1948), while significant genome transcription is first detectable at the 4 cell stage (Tomanek et al., 1989). The sixteen-cell morula-stage embryo enters the uterus on day 3, p. e., and by day 6 it has become a 64 cell blastocyst. Hatching from the *zona pellucida* takes place on days 6-7 (Prather et al., 1993). The embryonic disc is visible in a day 8 blastocyst. On days 10 to 11, p. e., the conceptus undergoes a rapid elongation phase. During this process, the conceptus changes its shape within hours, reaching a size of about 1 m in length. On day 15, elongated conceptuses begin to shorten to about 20-30 cm, thereby spacing themselves evenly along the porcine long uterine horns (King, 1993).

Blastocyst Implantation

Blastocyst implantation is a necessary event for establishment of pregnancy. By this process, the embryo becomes closely attached to the uterus in order to facilitate the exchange of nutrients, gases and waste products with the mother. Successful implantation requires differentiation of the uterus to a receptive state and is under the influence of steroid hormones (Mossman, 1987). Implantation can occur during a species-specific window of time when the activated stage of the blastocyst is superimposed on the receptive state of the uterus. This "implantation window" lasts for about 24 hours after day 4, p. e. in the mouse (Paria et al., 1993), and from days 13 to 18, p. e. in pigs (Roberts et al., 1993). In pigs, implantation starts on days 13-14, and placental changes continue until about mid-pregnancy (Dantzer, 1985). Swine and other ungulates exhibit epitheliochorial placentation. Unlike in primates or rodents, the ungulate endometrium is not subject to erosion by the embryonic membranes, resulting in the least invasive type of placentation (Mossman 1987).

Of an average of 15 fertilized ova per sow, only 60% result in live-borne piglets. The greatest reason that the remaining 40% of embryos do not survive to term is implantation failure, which accounts for 30% of embryonic losses (Pope, 1994). The majority of blastocysts that do not implant successfully are thought to be genetically normal and would probably survive if their activation was more synchronized with the receptive stage of the uterus (Roberts et al., 1993). It has been calculated that an increase in litter size would have the greatest impact on the economy of pig farming, greater than that due to increases in the rate of weight gain, lean/fat ratio, or obtaining a younger age at puberty (Tess et al., 1983).

Endometrium

The endometrium is a lining of the uterus that consists of epithelium and stroma, and is adapted for providing nourishment to the developing embryo and fetus. In some species, such as the human and mouse, there are specific regions of the endometrium where the placenta attaches more intimately (Mossman, 1987). Other species, such as ruminants, have specific structures, termed placentomes, where placental contact with endometrium is closest. In pigs, the entire endometrium has an equal capability to nourish the embryo (King, 1993).

Placentation

At days 12-13, p. e., a progressive interaction between the outer membrane of embryonic vesicles (chorion) and the uterine lining is initiated. Conceptus chorionic villi interdigitate with uterine epithelial folds (Keys and King, 1990). As attachment progresses, another membrane, the allantois, enlarges and joins the chorion to create the chorioallantoic membrane. The proliferating chorioallantoic membrane becomes corrugated over uterine glands, forming areolae to increase the surface area for absorption of glandular secretions. The placenta is fully formed by day 30 of pregnancy, but the contact area increases until about midgestation (King, 1993).

Unlike in primates and rodents, which exhibit two different invasive types of placentation (hemochorial and endotheliochorial, respectively), in swine and other ungulates the fetal and maternal tissues remain separate and intact throughout pregnancy. Embryos adhere to the endometrium by interdigitation between chorioallantoic membrane and endometrium, but do not penetrate the uterine wall. Among ungulates, and mammals in general, the *Suidae* exhibit the least invasive type of placentation. The shape of the porcine placenta is diffuse, i. e., chorionic villi are distributed over the entire surface of the chorionic sac (King, 1993).

Signaling Between Uterus And Embryo

Before implantation, both the uterus and embryo undergo substantial biochemical and morphological changes. These changes are synchronized, in part, by various signaling molecules present in ULF during the periimplantation period. Molecular signals, produced by the embryo and uterus, can stimulate or inhibit activities of one another, acting in a paracrine fashion, or can act in an autocrine manner upon the cells that synthesized them (Simmen et al., 1995).

Many molecules have been found in ULF, and attributed to signaling processes between the conceptus and its mother. These include the steroid hormones estrogen (E_2) and progesterone (P_4) (Roberts et al., 1987); growth factors and their binding proteins: insulin-like growth factor (IGF) -I and -II, IGF binding proteins (IGF/BPs), epidermal growth factor (EGF) (Simmen et al., 1995); and cytokines: interleukin 2 (IL-2), IL-6, tumor necrosis factor alpha (TNF-α), transforming growth factor beta (TGF-β) and leukemia inhibitory factor (LIF) (Hill, 1992; Mathialagan and Roberts, 1994). Conceptuses also contribute to the composition of ULF, via their secretion of biomolecules. On day 11, p. e., the level of estrogen in ULF is elevated due to synthesis by conceptuses (Bazer and Thatcher, 1977). Day 14 porcine trophoblast cells produce factors that activate cultured porcine macrophages, as determined by macrophage uptake and incorporation of tritiated thymidine into DNA (Ramsoondar et al., 1993).

Maternal Recognition Of Pregnancy

Changes during the reproductive cycle continue unless fertilization has occurred and the conceptus is present within the uterus. Conceptuses of various mammalian species signal their presence to the mother, i. e. elicit maintenance of the corpus luteum to ensure continued production of progesterone. Although the molecular signals can vary across species, the effect is the same: prostaglandin $F_2\alpha$ will reach the ovaries to maintain progesterone (P_4) release from the ovaries, and maintain pregnancy (Bazer, 1992). In pigs, the signal for maternal recognition of pregnancy are estrogens, secreted by the embryo around day 11, p. e. (Bazer and Thatcher, 1977). It is believed that four embryos within a uterus can produce enough estrogens for maternal recognition of pregnancy to occur (Bazer, 1992).

Steroid Hormones

Steroid hormones are involved in growth, developmental and differentiation processes of many different cell types (Truss and Beato, 1993). Steroids are hydrophobic molecules of low molecular weight (around 300). They travel through the blood bound to carrier proteins and enter cells by simple diffusion (Gorski and Gannon, 1976). Inside the cell, they bind to specific protein receptors via the hormone binding domain (HBD), thus changing the configuration of the DNA binding domain (DBD) of the receptor. When the configuration is changed by specific steroids, the receptor-ligand complex binds to one or more response elements in the target genes' regulatory region (Miesfeld, 1989). This binding stimulates or inhibits transcription of target genes. Transcriptional regulation by steroid receptors is the primary response to the presence of steroid. Products of the genes activated by steroids may in turn activate other genes, causing a secondary response (Baniahmad and Tsai, 1993).

Estrogens

Estrogens are considered to be the major implantation signals produced by the porcine blastocyst (Bazer and Thatcher, 1977), causing a thinning of endometrial epithelial glycocalyx and reduction of cationic ferritin binding to the *villi* of the endometrial epithelium. These changes are thought to be critical events for interdigitation of chorioallantoic and uterine *villi*, as well as for the adhesion between uterus and embryonic cells, necessary for establishment of pregnancy (Blair et al., 1991). Timing of estrogen synthesis, as well as of other signals, is extremely important for implantation and pregnancy. The peak estrogen synthesis by porcine embryos takes place on days 11 or 12 (Pusateri et al., 1990). However, if estrogen is administered to the sow before the blastocysts are competent (day 9 or 10), the "implantation window" of the uterus will "open" too early and the blastocysts will have less chance to attach (Morgan et al., 1987).

Steroid Synthesis

Cholesterol, the precursor for steroid hormones, is stored as an ester. It is taken up by endocytosis of low density lipoproteins. Adrenocorticotropic hormone (ACTH) activates cholesterol esterase via a cAMP - mediated phosphorylation event, resulting in increased free cholesterol in cells. Cholesterol's side chain is then cleaved in three successive monooxigenations, the final one causing C20 - C22 bond cleavage, resulting in pregnenolone (Jefcoate, 1986). Synthesis of steroid hormones is catalyzed by steroidogenic enzymes. The amount of a steroid hormone synthesized by a tissue depends on the steroidogenic enzyme level, steroidogenic enzyme activity, substrate availability and the number of specialized cells that may synthesize steroids upon induction by hormones. In the pathways of steroid hormone biosynthesis there are seven enzymes, located within two cellular compartments: P450scc and P450c11 are present in the inner mitochondrial matrix, whereas P450c17, P450c21, 3b-HSD, 17b-HSD and P450arom are in the membrane of the endoplasmic reticulum (Hanukoglu, 1992). These enzymes are members of the cytochrome pigment 450 (P-450) family. They are named after their unusual spectroscopic property to show a Soret absorption band at a wavelength of around 450 nm when oxidized. When reduced, they have maximum absorption at about 410 nm. There are more than one hundred known members of this family. Other members are involved in the metabolism of xenobiotics, biosynthesis of bile acids and vitamin D in liver and a myriad of other metabolic pathways (Negishi et al., 1993).

Growth Factors

Growth factors are multifunctional proteins that can stimulate or inhibit cell proliferation and/or differentiation, ultimately resulting in altered synthesis of proteins with regulatory, structural, and perhaps, nutritional functions (Simmen et al., 1995). Around the implantation period, the embryos of various species may synthesize one or more of the following growth factors: EGF, TGF-β, IGF-I and IGF-II, IGF-BPs I through 6, and estrogens. On the other side, uterine cells produce IGF-I, IGF-II, IGF-BPs, EGF, steroid receptors, prostaglandins and IGF-I receptors (Hofig et al., 1991). These molecules may mediate the interactions between the uterus and embryo. For example, IGF-I exhibits mitogenic effects in rat endometrial cells (Beck and Garner, 1992).

Cytokines

General

Cytokines are soluble polypeptides, which mediate immunological, inflammatory and reparatory tissue responses and interactions between cells. These proteins are synthesized primarily by immune cells, but are also produced by other non-lymphoid cell types. Cytokines can affect growth, mobility, and differentiation of many cells by acting in paracrine or autocrine fashion. Cytokines include interleukin-1 (IL-1) through IL-18, produced by monocytes and macrophages, and are involved in signaling between immune cells; TNF-α, released by macrophages and related to lymphotoxins of activated T-cells; GM-CSF; and colony stimulating factors, that control differentiation of hematopoietic stem cells. Interferons are mediators which increase resistance to viruses (La Bonnardiere,

1993) but can also induce steroidogenesis (Yasuda et al., 1992) and are mediators of the maternal recognition of pregnancy in ruminants (Thatcher et al., 1995).

Roles in reproduction. Cytokines are synthesized and secreted by the uterus and conceptuses of many species. Some biological effects of cytokines in reproduction were convincingly demonstrated, such as for LIF-deficient mutant mice (Stewart et al., 1992), some are only speculated upon, while others remain elusive. It is likely that the roles of most cytokines are overlapping, and that there is redundancy in the cytokine regulatory systems. In this regard, IL-6-deficient transgenic mice readily reproduce (Ramsay, 1994). IL-6 was found in the ULF of pregnant sows, with peaks at days 4 and 11, p. e. (Mathialagan et al., 1992). LIF is present in porcine ULF on day 12, p. e. (Anegon et al., 1994), while IFN-γ peaks at day 16, p. e. (La Bonnardiere, 1993). Besides leukocytes, other cell types, such as endometrial luminal and glandular epithelium, synthesize and secrete cytokines, potentially targeting macrophages, as well as trophoblast to regulate growth, differentiation and secretory activity (Guilbert et al., 1993).

Immunosuppression. During the periimplantation period, macrophages and neutrophils accumulate in the mouse uterus at sites of implantation where they produce cytokines. This process thus resembles a classical inflammatory reaction (McMaster et al, 1993). The role that was first ascribed to cytokines in the uterus was suppression of immune reactions by the mother, in response to the fetal allograft (Nicholas and Panayi, 1985; Clark et al., 1986). Not only maternal tissue, but also the trophoblast, produces cytokines and their receptors. On the basis of morphology, secretory activity and pluripotency, the trophoblast has been compared to macrophages (Guilbert, 1993), although a porcine trophoblast cell line does not produce major histocompatibility (MHC) class I and class II antigens, a unique feature of trophoblast in many species (Ramsoondar et al., 1993).

Steroidogenesis. Cytokines may also affect pregnancy events by regulation of steroidogenic enzyme gene expression. *In vitro* studies demonstrate that cytokines can either stimulate or inhibit steroidogenesis on at least three levels: by regulating the synthesis or activity of P450scc, P450c17 and $P450_{arom}$. Stimulation of P450scc synthesis causes increased pregnenolone levels (Jefcoate, 1986). Regulation at the level of P450c17 results in altered ratios of gluco- and mineralocorticoids relative to the sex steroids androgens and estrogens (New et al., 1984), while stimulation of P450arom increases the estrogen levels (Miller, 1988). Corticosterone production by cultured rat adrenal cells, was stimulated by IL-1 and IL-2, with concomitant increases in cAMP and PGE_2 (Tominaga et al., 1991). Injection of IL-1, IL-2, IL-6 and TNF-α *in vivo* resulted in increased blood levels of glucocorticoids (Hermus and Sweep, 1990). IL-1, IL-6 and TNF-α were detected in uteri of pregnant (De et al., 1993) and pseudopregnant (Choudhuri and Wood, 1993) mice, from 12 hours to 3 days, p. e. Both studies detected peaks in the cytokines' bioactivity at days 4-8, p. e., with IL-1 peaking at days 4 and 5, IL-6 at days 5 and 6, and TNF-α at day 8, p. e., respectively. Progesterone synthesis by trophoblast was positively regulated by IL-1 and TNF-α (Haimovici et al., 1991; Feinberg et al., 1994), but was negatively regulated by IFN-γ in luteinized granulosa cells (Fukuoka et al., 1992). IL-1, IL-2, TNF-α and IFN-γ inhibit luteotrophic hormone induced androgen synthesis in rat, mouse and porcine testicular Leydig cells and in ovarian theca interna cells. These factors probably inhibit CYP17 transcription, which is coupled with a decrease in human chorionic gonadotropin - (hCG) supported accumulation of cAMP

(Orava et al., 1989; Andreani et al, 1991; Hurwitz et al., 1991; Hales, 1992). Estrogen synthesis was inhibited by IL-1, IL-2, TNF-α and IFN-γ, in luteal cells (Fairchild and Pate, 1991; Wang et al., 1992; Pitzel et al., 1993) and luteinized human ovarian granulosa cells (Fukuoka et al., 1992).

Implantation. *In vivo* studies on the pregnant mouse demonstrated that fetal losses associated with the antiphospholipid syndrome were prevented by administration of murine recombinant IL-3 (Fishman et al., 1993). LIF was found in ULF of day 4 pregnant mice (Bhatt at al., 1991), and its reproductive role was clearly demonstrated using null mutant mice, deficient in LIF production. These mice conceive, but their embryos fail to implant. However, embryos of LIF-deficient mice can implant successfully and be carried to term, if transferred to a wild-type, pseudopregnant mouse, thereby suggesting that uterine-produced LIF is essential for pregnancy establishment in this species (Stewart et al., 1992).

Hematopoietic cytokines

LIF and IL-6 are members of the hematopoietic cytokines group, which includes IL-11, oncostatin M (OSM), ciliary-neurotropic factor (CNTF), granulocyte colony stimulating factor (G-CSF), erythropoietin (EPO) and cardiotrophin-1 (CT-1) (Peters et al., 1995). All IL-6-type cytokines, including LIF, as well as IL-11, G-CSF, OSM, CNTF, cardiotropin-1 (CTP-1), EPO, utilize a common 130 kD glycoprotein (gp130) receptor subunit. In addition to gp130, those cytokines bind to their specific receptor subunit (Hibi et al., 1990; Bellido et al., 1996). The biological effects of LIF and IL-6 include a broad spectrum of physiological changes caused by altered expression of other genes. These two cytokines act through several transcription factors including members of the C/EPB family, as well as JunB, and the glucocorticoid receptor (Baumann et al., 1992). Some effects overlap, while others are specific for one or the other cytokine (Piquet-Pellorce et al., 1994).

Hematopoietic cytokines regulate hematopoietic and other cell functions via specific cell surface receptors. These receptors are members of a large superfamily of transmembrane proteins and are characterized by a 200 amino acid extracellular sequence encoding the ligand binding domain. There is a highly conserved organization between the members of this superfamily. The LIFR-β locus is present in a cluster of receptor genes on human chromosome 5, suggesting the possibility that subsets of cytokine receptor genes may be organized into clusters (Baird et al., 1995).

Cytokine receptors that are gp130-associated can be grouped into signal transducing and non-transducing classes. Together with the fact that there is primary sequence homology between cytokines of the hematopoietic cytokine group, there is a basis for the hypothesis that these have evolved by repetitive duplications of cytokine and receptor. These receptors may have arisen from a common ancestral complex which included a homodimer of two gp130-like signaling receptors and an IL-6R-α-like non-signaling receptor. Alterations in the components of the group are proposed to have arisen by receptor duplication and divergence to allow signal transduction via a LIF receptor-beta (LIFR-β)/gp130 heterodimer, and loss of the non-signaling receptor component in the G-CSF and LIF lineages (Shields et al., 1995).

LIF, OSM, and IL-6, and probably other members of the group, utilize the Janus kinase (JAK) family of cytoplasmic tyrosine kinases. The beta receptor components for this cytokine family, namely gp130 and LIFR-β, constitutively associate with the JAK/TYK kinases. Activation of these kinases occurs as a result of ligand-induced dimerization of the receptor beta components. The receptors for this cytokine family utilize all known members of the JAK-TYK family (Stahl et al., 1994). LIF and IL-6 have been implicated in reproductive processes in mouse and other species, such as embryo implantation (Bhat et al., 1991; Stewart et al., 1992), growth and development (Murray et al., 1990) and differentiation (Shen and Leder, 1992).

Leukemia inhibitory factor

History. There has been much confusion in nomenclature with this pleiotropic cytokine being called several different names as a consequence of its different functions. The consensus name comes from the ability of LIF to inhibit differentiation of myeloblastic leukemia cells. Other names (explaining functions) that have been used include: Human interleukin for DA cells (HILDA), Differentiation inhibiting activity (DIA), Differentiation inducing factor (D-factor), cholinergic neuronal differentiation factor (CDF), and hepatocyte-stimulating factor (HSF-III) (Metcalf et al., 1991).

Structure. The human mature LIF glycoprotein is 36-67 kD in size, and consists of 180 amino acids (Moreau et al., 1988). X-ray crystallography revealed the four antiparallel alpha-helix bundle topology, and the closest structural homology to G-CSF, growth hormone (GH) and IL-6 (Bazan, 1991). There are 2 forms of LIF: diffusible (D-LIF) and matrix associated (M-LIF), differing at the 5' ends of their messenger RNA (mRNA) (Rathjen et al., 1990). Analysis of the biological functions and receptor specificity of a series of human-mouse LIF chimeric proteins implicated two regions for receptor interaction, located in the fourth helix and the preceding loop, respectively (Robinson et al., 1994). In the human, the LIF gene was mapped to chromosome 22 (Rose and Bruce, 1991). Porcine LIF gene was cloned by Wilson et al. (1992).

Regulation. In human lung fibroblasts and in umbilical vein endothelial cells, LIF production was induced by IL-1-β, TNF-α and phorbol esters (Lubbert et al., 1991). Phorbol myristate acetate (PMA), an activator of protein kinase C, is a potent stimulator of LIF, while dexamethasone inhibited PMA and IL-1β induction of LIF in human cartilage cells (Lotz et al., 1992). In human endometrial stromal cells, IL-1, TNF-α, platelet-derived growth factor (PDGF), EGF and TGFβ were potent inducers of LIF expression, while IFN-γ inhibited LIF expression induced by these cytokines. In endometrial glandular epithelium, LIF expression was constitutive (Arici et al., 1995). As for many other reproductive events, there is also evidence for steroid hormonal regulation of LIF. LIF mRNA has been amplified by reverse transcription-polymerase chain reaction (RT-PCR) from progesterone- but not estrogen-dominated endometrium of the rhesus monkey (Ace and Okulicz, 1995). These findings provide evidence for hormonal regulation of LIF that may underlie the normal maturation of the primate endometrium in preparation for implantation. Therefore, it is possible that the LIF gene may be regulated by steroids. This assumption is supported by the analysis of the mouse LIF gene, in which an upstream regulatory region (between -3200 and -1200) contains sequences related to minimal steroid responsive elements (Hsu and Heath, 1994).

Biological effects. LIF can inhibit or stimulate differentiation of tumor cells, depending on the tumor type. It inhibits differentiation of myeloblastic leukemia cells, but can stimulate proliferation of human breast tumor cells, which express functional LIFRs

(Estrov et al., 1995). LIF also acts as a paracrine or autocrine growth factor for breast, kidney and prostate tumor cells, in the presence or absence of estrogen (Kellokumpu-Lehtinen et al., 1996). Both LIF and IL-6 play a role in the acute immune response by stimulating type-2 acute phase plasma protein production in response to infection (Baumann et al., 1992). LIF induces the synthesis of other molecules associated with inflammation, such as cyclooxygenase II in human chondrocytes (Geng et al., 1995), as well as $\alpha_2$macroglobulin in astrocytes in the presence of glucocorticoids (Higuchi et al., 1994). Some other targets of LIF are cells of nervous and bone tissues. There is an important role for LIF in neural development, particularly as a differentiation factor. LIF induces acetylcholine in sympathetic neurons, therefore differentiating adrenergic neurons into cholinergic ones (Vandenbergh et al., 1991). LIF also prevents expression of muscarinic receptors in cultured sympathetic neurons (Ludlam and Kessler, 1993). Mice lacking LIFR-β exhibit a serious loss in their motor neurons and die shortly after birth unlike mice lacking CNTF or LIF, which are viable, indicating a role of LIF signal in neuronal differentiation. (Li et al., 1995).

Mode of action. LIF acts upon its target cells through interacting with the LIFR. The receptor is structurally and functionally similar to IL-6R, except that the specific, low affinity subunit is a 190 kD glycoprotein (gp190), also called LIF binding protein or LIFR-β. The other subunit is a gp130, a high-affinity signaling subunit. The binding of ligand induces heterodimerization of gp190 and gp130 (Davis et al., 1993), and the dimer activates Janus kinase (JAK) (Lutticken et al., 1994). The activated receptor-kinase complex recruits signal transducer and activator of transcription-3 (STAT-3) and activates it by phosphorylation. As a consequence, the phosphorylated STAT-3 protein dimerizes, translocates into the nucleus, binds response elements in the promoter region of target genes and stimulates gene transcription (Heim, 1996; Minami et al., 1996). This is referred to as the JAK/STAT pathway. Signaling activities of IL-6, IL-11, CNTF, OSM and LIF can be specifically blocked by different anti-gp130 monoclonal antibodies, suggesting that there are regions on gp130 where cytokines bind or interact specifically (Wijdenes et al., 1995). A myeloid leukemia cell line (M1) has been established that constitutively expresses STAT-3. Unlike normal M1 cells, the differentiation of these cells could not be induced by LIF or IL-6. Growth arrest and apoptosis could not be induced either, suggesting that STAT-3 activation is the critical step in a cascade of events that lead to terminal differentiation of M1 cells (Minami et al., 1996).

Role in reproduction. LIF was previously implicated in reproductive processes: implantation (Bhat et al., 1991; Stewart et al., 1992), and growth and development (Murray et al., 1990) as well as differentiation (Shen and Leder, 1992) of mammalian embryos. The culture of mouse embryonic stem (ES) cells requires a feeder layer of fibroblasts to keep the ES cells in an undifferentiated state. A feeder layer can be replaced by addition of LIF into the culture medium (Williams et al., 1988). It is now established that LIF inhibits the differentiation of mouse (Pease et al., 1990) and pig (Hochereau de Reviers and Perreau, 1993) embryonic stem cells, and induces proliferation of mouse trophoblast (Lavranos et al., 1995). The suppression of embryonic stem cell differentiation by LIF may take place via cyclin E/CDK2 complexes, p21Cip1 and p27Kip1 cyclin-dependent kinase inhibitors and cyclin D/CDK4. The expression of these was suppressed in mouse embryonic stem (ES) cells exposed to LIF. When LIF was removed, all genes were up-regulated at the mRNA level (Savatier et al., 1996). LIF, but not IL-6, promoted the proliferation of primordial germ cells (PGC) and enhanced their viability *in vitro*. Addition of neutralizing antibodies against gp130 in culture, blocked PGC survival, suggesting a pivotal role of gp130 in PGC development (Koshimizu et al., 1996). LIF is essential for mouse conceptus implantation (Stewart et al., 1992), and was found to be expressed at the time of implantation in endometrium of the human (Charnock-Jones et al., 1994), mouse (Bhatt et al., 1991), rabbit (Yang et al., 1994), pig (Anegon et al., 1994), and cow (Brisson et al., 1996). It is not known if LIF is essential for implantation of the human conceptus. However, there is an increasing body of evidence to suggest that this is the case. LIF is predominantly expressed in the endometrial glands of the human endometrium, most abundantly during the secretory/postovulatory phase, and less during proliferative/preovulatory phase, coincident with the time of implantation (Arici et al., 1995). LIFR-β is expressed predominantly by the luminal epithelium during both phases of the menstrual cycle. Gp-130 is expressed in both the luminal and glandular epithelium throughout the cycle (Cullinan et al., 1996). Therefore, LIF may have a role during the implantation processes of the human conceptus, possibly through an autocrine/paracrine interaction between LIF and its receptor at the luminal epithelium. LIF suppressed trophoblast production of human chorionic gonadotropin (hCG) protein, as well as expression of beta hCG mRNA (Nachtigall et al., 1996). LIF, as well as IL-6, IL-11 and OSM significantly inhibited gonadotropic hormone releasing factor (GHRF) secretion by cultured mouse placental cells (Yamaguchi et al., 1995). LIF increased the expression of oncofetal fibronectin mRNA and secretion of the protein, but did not affect progesterone production (Nachtigall et al., 1996). Thus, LIF appears to be an important regulator of human embryonic implantation by directly modulating trophoblast differentiation. LIF stimulates mouse placental lactogen I (mPL-I) secretion by cultured placental cells from day 9 of pregnancy, but inhibits mPL-II secretion by days 7, 9, and 12 placental cells (Yamaguchi et al., 1995). King et al. (1995) were unable to amplify by PCR the LIF mRNA from human villous and extravillous trophoblast cell populations, although LIFR was present in both cell populations, suggesting that LIF may be transported to this site of action. There are indications that LIF is involved in the hypothalamic-pituitary-adrenal/gonadal axis. LIF mRNA has been detected by Northern hybridization of cultured pituitary explants from all three lobes of the rat pituitary (Carter, 1995). Moreover, LIF exhibits effects on AtT20 mouse pituitary cells, namely increased proopiomelanocortin (POMC) and ACTH synthesis, both of which can be inhibited by anti-LIFR-β or anti-gp130 antisera (Ray et al. 1996). LIF appears to have a role in tissue remodeling, or its prevention, which may be important to pregnancy outcome. The mechanism of trophoblast invasiveness may depend on the expression of metalloproteinase gelatinase B (MMP-9) and of matrix urokinase-type plasminogen activator (uPA), which are expressed in the mouse trophoblast. Expression of these enzymes in trophoblast was stimulated by the addition of LIF to embryo cultures (Harvey et al., 1995). In humans, trophoblast invasiveness *in situ* may be controlled by the local production of anti-invasive factors by the uterus. One of these factors is tissue inhibitor of metalloproteinases-I (TIMP-1), which neutralizes metalloproteases (Graham and Lala, 1992). Inhibitors of metalloproteinases are stimulated by LIF and may have a similar role in mouse uterus (Harvey et al., 1995). LIF may also play a role in the paternal side of reproduction. LIF-

and CNTF-like proteins of the expected molecular masses were found in testicular extracts of 3-day-old rats. LIF significantly enhanced the survival, but not proliferation, of the Sertoli cells in a dose- and time-dependent manner. It enhanced both survival and proliferation of gonocytes, indicating that LIF or CNTF may have a role in initiation of spermatogenesis (De Miguel et al., 1996).

Interleukin-6

History. The first observed effect of IL-6 was stimulation of differentiation of B-cells into immunoglobulin-producing cells. Therefore, it was also known as B-cell stimulating factor 2 (BSF-2) (Kawano et al., 1988). Other synonyms are hepatocyte stimulating factor; hybridoma plasmacytoma growth factor and interferon beta II (IFN-β_2) (Yasukawa et al., 1987).

Structure. Human IL-6 cDNA was cloned by Yasukawa et al. (1987). This is a 184 amino acid long protein (Richards and Saklatuala, 1991) that migrates on SDS-PAGE gels as 23 to 30 kD bands (May et al., 1988). The heterogeneity in size may be a consequence of differential RNA splicing, since Northern analysis revealed a predominant 1.3-kb transcript and several minor transcripts between 1.9 and 4.8 kb (Kestler et al., 1995). Human IL-6, similar to LIF, has an antiparallel 4 helix-bundle conformation (Bazan, 1991). Porcine cDNA was cloned by Mathialagan et al. (1992).

Regulation. Expression of IL-6 gene and protein is positively affected by IL-1, TNF-α (Norms et al., 1994; Rifas et al., 1995), PDGF (Franchimont and Canalis, 1995), OSM (Brown et al., 1991), and IL-4 (Donnelly et al., 1993). Nuclear factor-IL-6 (NF-IL-6) is a transcriptional activator, thought to be involved in the activation of the IL-6 promoter in response to IL-1 and bacterial lipopolysaccharides. Stimulation of the protein kinase C pathway leads to phosphorylation of Ser 105 within the activation domain of NF-IL-6, and enhances its transcriptional efficiency (Trautwein et al., 1993). IL-6 expression is also upregulated by NF-κB (Matsusaka et al., 1993), and the IL-6 gene was found to have NF-κB response elements in its 5' regulatory region (Kopp and Ghosh, 1994). The complement activation fragment C5a has been shown to induce IL-6 synthesis by peripheral blood mononuclear cells. Pretreatment of pigs with C5a peptide-specific monoclonal antibody (mAb), prior to induction of sepsis by *Escherichia coli*, results in a marked decrease of IL-6 bioactivity in serum (Hopken et al., 1996). In ovariectomized mice, systemic administration of the combination of estrogen and progesterone resulted in increased levels of IL-6 expression, indicating that IL-6 normally may be induced in uterus by ovarian hormones (De et al., 1992). Interleukin-1-beta (IL-1-β) converting enzyme (ICE) processes the inactive IL-1-β precursor to the proinflammatory cytokine. There is evidence that ICE may be required for processing of IL-6 since adherent monocytes from mice harboring a disrupted ICE gene did not export IL-1-α, interleukin-1-beta (IL-1-β), IL-6 or TNF-α (Kuida et al., 1995). Synthesis of IL-6 is negatively regulated by IL-4 and IL-10. The potential mechanism of IL-6 inhibition by IL-4, is binding inhibition of factors that stimulate IL-6 expression, i.e. activator protein-1 (AP-1) and NF-IL-6, but not of NF-κB. IL-10 inhibits binding of AP-1, but not of NF-IL-6 or NF-κB. Besides binding inhibition, IL-4 also downregulates the mRNA expression of the two major components of the AP-1 complex, namely c-fos and c-jun (Dokter et al., 1996). IL-6 is also negatively affected by phosphokinase C (PKC) pathway (Gross et al., 1993), p53 (Santhanam et al.,

1991) and 17β-estradiol (E_2) in uterine epithelial cells (Tabibzadeh et al., 1989; Jacobs et al., 1992). The IL-6 inhibition by E_2 is mediated by the transcription factors NF-κB and NF-IL-6 (Stein and Yang, 1995). Besides the transcriptional level, regulation of IL-6 biosynthesis can take place at the translational level, at least in certain pathological conditions. During infection with two species of *Shigella spp.*, human rectal biopsic specimens exhibited 3- to 100-fold increases in IL-6 mRNA, but the immunohistochemical staining revealed no changes in IL-6 synthesis (Raqib et al., 1996).

Biological effects. IL-6 is highly expressed in liver nuclei, where it is also considered to be a regulator of the acute-phase immune response to infection. The acute-phase immune response to infection is mediated by acute-phase reactants (APRs), proteins which are capable of binding the bacterial cell wall product endotoxin (Baumann et al., 1992). One of APRs is lipopolysaccharide-binding protein (LBP). The LBP promoter includes regulatory elements, such as the common acute-phase response element (APRE), to which acute-phase response factor (APRF)/STAT-3 binds (Schumann et al., 1996). Since IL-6 rapidly activates APRF/STAT-3 by tyrosine phosphorylation (Lutticken et al., 1994), the mechanism of IL-6 induction of acute-phase immune response may be indirect, via APRF/STAT-3 upregulation of the expression of APRs. IL-6 induces human C-reactive protein (CRP) (Li and Goldman, 1996), another APR, which induces production of T- (thymus-derived) lymphocytes (Croft et al., 1976). Some antiinflammatory drugs break the cascade of events leading to APR activation. For example, aspirin inhibits activation of NF-κB, which induces IL-6 transcription, thereby indicating that inhibition of IL-6 transcription may be one mechanism for the anti-inflammatory actions of aspirin (Kopp and Ghosh, 1994). Besides inducing the acute phase response, IL-6 has been associated with a myriad of biological effects. For example, IL-6 is one of the factors involved in allograft rejection (Krams et al., 1993) and plays a role in angiogenesis by stimulating endothelial cell proliferation (Motro et al., 1990). It is thought that IL-6 also plays a role in hematopoiesis, since stimulation of purified human CD34+ erythroid cells with IL-6 and soluble IL-6R-α (sIL-6R-α), stimulates proliferation, differentiation and maturation of these cells. The addition of anti-gp130 monoclonal antibodies to these cultures completely abrogated the generation of erythroid cells (Sui et al., 1996). A knockout mouse line has been generated via disruption of the IL-6 gene by homologous recombination. These mice have severely compromised T-cell-dependent antibody responses, as well as diminished inflammatory acute-phase responses after tissue damage or infection. The inflammatory acute-phase response of these mice was only moderately affected after challenge with lipopolysaccharide (Kopf et al., 1994). Mice without a functional IL-6 have reduced number of immunoglobulin A (IgA)-producing cells, resulting in deficient local antibody responses. However, intranasal infection with recombinant *Vaccinia* virus expressing IL-6, restored the IgA response in lung tissue (Ramsay et al., 1994). IL-6 is required for the synthesis of immunoglobulin class E (IgE) by peripheral blood mononuclear cells. Since IgE is involved in the allergic reaction, IL-6 may play a role in allergies (Vercelli et al., 1989). IL-6 is involved in the regulation of B cell response into antibody producing cells, and it also acts upon T cells, hematopoietic stem cells, hepatocytes, nerve cells and myeloma cells (Matsuda et al., 1989). Myeloma cells produce IL-6 and express its receptors, and anti-IL-6 antibody inhibits the in vitro growth of myeloma cells, suggesting that IL-6 is a part of an autocrine loop that is operative in oncogenesis of human myelomas (Kawano, et al., 1988). Both IL-6 and LIF can induce cyclooxygenase II in human chondrocytes (Geng et al., 1995), as well as $\alpha_2$macroglobulin in astrocytes in the presence of glucocorticoids (Higuchi et al., 1994). The concentration of vascular endothelial growth factor (VEGF) mRNA is increased in response to IL-6 in various cell lines (Cohen et al., 1996). A similar stimulatory effect of IL-6 was observed with APR hemopexin in rat hepatoma H-35 cells (Immenschuh et al., 1995). IL-6 also stimulated c-fos and c-fms, but inhibited c-myc expression in the human myelomonoblast line THP-1 (Brach et al., 1990). IL-6, in combination with dexamethasone, induced pancreatitis-associated protein synthesis in pancreatic acinar cells (Dusetti et al., 1995), while inhibited IL-5 in normal human lymphocytes (Enokihara et al., 1994).

Mode of action. In order to elicit biological actions, IL-6 has to be internalized into the cell. A 10 amino acid sequence within the intracellular domain of gp130 was identified as being crucial for internalization of IL-6. Deletion analysis of this region showed that a di-leucine pair was essential for ligand internalization. Three serine residues upstream of this region can further enhance internalization (Dittrich et al., 1996). IL-6 acts upon target cells via the IL-6 receptor (IL-6R). Human IL-6R consists of two components. One is the 80 kD glycoprotein (gp80), termed IL-6R-α (Taga et al., 1989), which is specific for this ligand and has low affinity. It can be trans-membrane or soluble. IL-6R-α cDNA of 468 amino acids has been cloned and characterized by Yamasaki et al. (1988). It includes a signal peptide of approximately 19 amino acids and a domain of approximately 90 amino acids that is similar to a domain in the immunoglobulin (Ig) superfamily. The other receptor component is gp130, that consists of 918 amino acids in humans (Hibi et al., 1990). IL-6 - IL-6R-α complex binds gp130, with high affinity. Gp130 is shared by other hematopoietic cytokine receptors, such as those for LIF, CNTF, OSM, and IL-11. The binding of ligand to the IL-6R-α induces homodimerization of two gp130 molecules. Tyrosine kinase activation by homodimerized gp130 depends on proline residues 656 and 658 in the cytoplasmic motif of gp130 (Zhong et al., 1994). Gp130 dimer associates with members of JAK/TYK family of tyrosine kinases thereby activating them (Murakami et al., 1993; Lutticken et al., 1994; Wang and Fuller, 1994). Thus, a general first step in IL-6-related cytokine signaling may be the dimerization of signal-transducing molecules and activation of associated tyrosine kinases. Unlike other studied cytokine receptors, these for the hematopoietic cytokines family utilize all known members of the JAK/TYK family, but can induce distinct patterns of JAK-TYK phosphorylation in different cell lines or types (Stahl et al., 1994). Activated JAK/TYK catalyze phosphorylation of APRF/STAT-3 on tyrosine residues, which become activated to bind DNA sequences, in the regulatory regions of other genes (Zhong et al., 1994; Lutticken et al., 1994). APRF/STAT-3 binding sites were identified in upstream regulatory regions of various genes, such as LBP (Schumann et al., 1996), junB (Fujitani et al., 1994; Coffer et al., 1995), human oxytocin receptor (Rozen et al., 1995), rat serine proteinase inhibitor 3 gene (Kordula and Travis, 1996) and IFNAR-1 chain of the human type I interferon receptor (Yang et al., 1996). The mechanism of IL-6 induction of gene expression may include other factors, like cytokine responsive elements (CyRE), which have been identified in the regulatory regions of the vasoactive intestinal peptide (VIP) gene (Symes et al., 1995). IL-6 induced the binding of nuclear factors to IL-6 response elements (IRE) flanking the junB gene promoter (Coffer et al., 1995). IL-6R components may be regulated in constitutive fashion or by other factors. In megakaryoblastic cells, mRNA for IL-6R-α was constitutively expressed (Sasaki et al., 1995). LIF downregulated IL-6R-α expression in mouse myelomonocytic leukemic M1 cells (Yamaguchi et al., 1992), and IL-6 induced the downregulation of IL-6R-α expression in myeloma cells (Lasfar et al., 1994). IFN-γ increased the IL-6R-α mRNA level, but treatment with IFN-γ together with TNF-α reduced the level of IL-6R-α mRNA expression in monocytic cells (Sanceau et al., 1991). Schoester et al. (1994) could not detect ligand-induced downregulation of IL-6R-α or IL-6R-β. Instead, they detected a slight upregulation of IL-6R-β by IL-6 in primary human blood monocytes. Dexamethasone could up-regulate the number of IL-6R-α subunits on plasma membranes of human epithelial and hepatoma cell lines (Snyers et al., 1990). IL-6, IL-1 and TNF-α treatment enhanced gp130 mRNA expression in two human epithelial cell lines (Snyers and Content, 1992), while IL-6, administered together with dexamethasone, induced gp130 at the mRNA level (Schooltink et al., 1992). OSM stimulated, whereas IL-1 inhibited IL-6-α expression in rat hepatoma cells at the levels of mRNA (Geisterfer et al., 1995).

Role in reproduction. Alterations in immune activity are often accompanied by reproductive disorders. There are indications that cytokines play a role in these situations, which would suggest that they interfere with the hypothalamic-pituitary-gonadal axis. IL-6 is produced by rat anterior pituitary cells in culture (Spangelo et al., 1990) and IL-6R-α (Arzt et al., 1993) is expressed in the pituitary. In the anterior pituitary gland, IL-6 directly stimulates the release of adrenocorticotropic hormone (ACTH), prolactin and somatotropic hormone (STH) (Jones and Kennedy, 1993). IL-6 stimulated the release of corticotropin-releasing hormone-41 (CRH-41) in fresh rat hypothalamic explants (Navarra et al., 1991). IL-6, when applied as an intracerebroventricular (icv) injection, can lower plasma LH levels in castrated rats (Rivier and Vale, 1990). In this case, IL-6 probably acts upon the hypothalamus, which expresses IL-6R-α (Gadient and Otten, 1993). In general, it is thought that IL-6, together with IL-1, IL-2, TNF-α and IFN-τ, suppresses the hypothalamic-pituitary-gonadal axis, but stimulates the hypothalamic-pituitary-adrenal axis (Sweep et al., 1991; Jones and Kennedy, 1993). IL-6 is expressed in the mouse (Choudhuri and Wood, 1993) and porcine uterus (Anegon et al., 1994), as well as in porcine, ovine and bovine conceptuses around the time of implantation (Mathialagan et al., 1992). IL-6 is expressed in the human endometrium, at higher levels during mid-secretory and menstrual phases, while at lower levels during proliferative and early secretory phases (Tabibzadeh et al., 1995). IL-6R-α gene expression did not change throughout the porcine estrous cycle (Anegon et al., 1994). Human first-trimester decidualized endometrial tissues expressed IL-6 following culture in the presence of IL-2. This indicates that these cells could play a role in regulation of placental growth (Deniz et al., 1996). IL-6 inhibited mPL-II secretion by cultured mouse placental cells after midpregnancy. Addition of soluble IL-6R-α together with IL-6 results in an inhibition of mPL-II production before midpregnancy (Yamaguchi et al., 1995). A possible role for IL-6 in reproduction could be to enhance antibody presentation by epithelial and stromal cells of the uterus (Prabhala and Wira, 1995). This could be important for conferring resistance from infections within the reproductive tract. IL-6 may have a role in reproduction as a stimulator or suppresser of steroid hormone synthesis, which might be another link to reproductive events. Addition of IL-6 to granulosa cell cultures, including macrophage contaminants, resulted in stimulatory effects on basal and hCG-stimulated progesterone production and on basal and FSH-stimulated aromatase activities. Together with the fact that IL-6 is synthesized in granulosa cells, this study suggests that IL-6 might be an intraovarian regulatory factor concerned with steroidogenesis (Machelon et al., 1994). IL-6 has been found to stimulate cytochrome $P450_{arom}$ expression in human breast cancer cells (Reed et al., 1993). Most hematopoietic cytokines, including IL-6, were able to stimulate cytochrome $P450_{arom}$ expression in adipose tissue. This pathway involves a JAK/STAT signaling mechanism, acting together with glucocorticoids and the transcription factor Sp1 (Zhao et al., 1995). Mutant mice deficient in IL-6 synthesis can reproduce (Ramsay, 1994), therefore IL-6 is not essential for embryo implantation or pregnancy maintenance in this animal model. It is possible, however, that one or more other cytokines of the hematopoietic group activate the gp130-JAK/STAT pathway, so that there is redundancy in the system, like there is with the IGF system. IL-6 may play a role on the paternal side of reproductive processes as well. IL-6 is expressed in Sertoli cells (Syed et al., 1993) and germ cells, where it may play a role in the replication of meiotic DNA (Dugast and Jegou 1994).

Antileukoproteinase

History

Antileukoproteinase (ALP) is a lysosomal serine proteinase - inhibitor, secreted by mucosa. Its function was first associated with the inhibition of activities of the neutral serine lysosomal proteinases: elastase and cathepsin-G. It is also known as a secretory leukocyte protease inhibitory (SLPI)(Thompson and Ohlson, 1986).

Structure

Porcine ALP is a 114 amino acid protein encoded by an mRNA which is about 800 nucleotides long (Farmer et al., 1990). The human protein has two domains, involved in binding trypsin/cathepsin-G and elastase, respectively (Steffens et al., 1993). A study using sheep anti-porcine ALP antibodies revealed that the pALP in uterine flushings and allantoic fluid is a 14 kD protein, which exhibits elastase inhibitor activity (Simmen et al., 1992). The porcine ALP protein is encoded by a 13 kbp gene consisting of 5 exons and 4 introns (Simmen et al., 1993). An X-ray crystallography study revealed that SLPI has a boomerang-like shape consisting of two domains. Polypeptide segments of each domain are interconnected by four disulfide bridges. The binding loop of the first domain probably has anti-trypsin activity, while the reactive site loop of the second domain has elastase and chymotrypsin binding properties (Grutter et al., 1988).

Regulation

A TATA box is located at -29 nucleotides from the transcription start site. Potential binding site sequences for transcription factors: AP-1, AP-2, SP-1 and Oct-1 were identified in the 5' flanking region of the porcine ALP gene. A fragment of approximately 240 bp, most proximal to the transcription initiation site, confers basal and limited endometrial tissue-specific promoter activity to the ALP gene. The porcine gene has a signal sequence encoded by exon 1, binding regions for trypsin/cathepsin G in exon 2, and for elastase in exon 3. This organization is similar to that of the human gene, although the genomic sequence is about three times longer than for the human gene (Simmen et al., 1993). Progesterone and estrogen do not induce the expression of ALP mRNA in ovariectomized gilts (Simmen et al., 1991), but do increase the ALP mRNA levels in prepubertal gilts (Farmer et al., 1990). The presence of ALP protein varies with the stage of pregnancy, in endometrium, allantoic fluids, and fetal cord blood, but is unchanged in placenta, myometrium, and fetal liver (Simmen et al., 1992). Embryo-conditioned medium causes an increase in endometrial ALP mRNA and days 16 to 25 pregnant endometrium had significantly greater ALP mRNA levels than endometrium obtained from anti-mesometrial and inter-implantation sites (Reed et al., 1996b). Deletion of 5' regulatory regions revealed that sequences located between -887 and -243 bp of the ALP gene directed CAT gene expression in endometrial cell lines from three species (Simmen et al., 1993). In human airway epithelial cells, ALP gene is positively regulated by phorbol ester (Maruyama et al., 1994), IL-1 and TNF-α (Sallenave et al., 1994).

Biological Effects

ALP gene expression in porcine lungs is constitutive (Farmer et al., 1990). In humans, ALP has been proposed to protect the epithelial linings adjacent to mucous secretions against degradation by elastase and cathepsin-G (Vogelmeier et al., 1991). A specific form of ALP, elafin, is expressed in human skin. Elafin is an elastase specific inhibitor, which is thought to play a regulatory role in inflammation (Molhuizen and Schalkwijk, 1995). In skin, its level of expression is elevated during some skin diseases, such as psoriasis (Schalkwijk et al., 1991).

Mode of Action

The carboxyl terminal part of ALP is thought to be responsible for its proteinase inhibitory activity (Thompson and Ohlsson, 1986; Eisenberg et al., 1990), while the amino terminal domain is believed to be involved in binding to proteinases (Ying et al., 1994). SKALP, a protein related to ALP has the ability to bind to cathepsin-G and elastase, blocking the reactive serine at the catalytic center (Molhuizen and Schalkwijk, 1995).

Role in Reproduction

ALP is expressed by the pregnant porcine endometrium. The mRNA levels are low in early pregnancy, and high in mid and late pregnancy, (Farmer et al., 1990), suggesting a possible pregnancy-dependent role for ALP in feto-maternal interactions. Radioimmunoassays showed the presence of ALP protein in early, mid, and late pregnant endometrium and myometrium, placenta, allantoic fluids, fetal cord blood, and fetal liver, although it was undetectable in the maternal circulation (Simmen et al., 1992). Badinga et al. (1994) measured the relative expression of ALP mRNAs and protein in the endometrium of horse, cow, mouse and rat, and found a correlation between relative ALP gene expression and the epitheliochorial placentation. Together with the previous reports of ALP expression in the pig (Simmen et al., 1991), and its absence in human endometrium (Heinzel et al., 1986), this finding supports the hypothesis of Samuel (1971), that the level of conceptus invasiveness depends on the endometrium rather than on the conceptus. It further suggests a functional role for this protein in the maintenance of the placental-uterine interface characteristic of species with epitheliochorial placenta (Badinga et al., 1994). Moreover, ALP protein was detected in porcine placenta, where ALP mRNA is absent, suggesting the transplacental transport of this protein (Simmen et al., 1991).

Beta$_2$microglobulin

History

Beta$_2$microglobulin (β_2m) is the light chain subunit of the major histocompatibility complex (MHC) antigens (Klein and O'hUigin, 1993). MHC antigens were first identified in connection with organ transplant rejection (Bjorkman et al., 1987). Since it is obvious that organ transplantation does not occur in natural conditions, the question arose as to what function MHC antigens might have in biological situations. At least two situations were later identified when MHC antigens were beneficial for the organism. One is in the case of tumors, when affected cells have mutated MHC antigens which can be recognized by the immune system (Hui, 1989), and the other is with viral infections, in which viruses may alter MHC expression, and thereby make host cells recognizable by the immune system (Townsend, 1987).

Structure

In swine, β_2m is a 119 amino acid, 11-kD protein (Milland et al., 1993). The light chain of the class I mouse MHC consists of β_2m, while the heavy chain is a 40- to 45-kD protein that consists of α_1, α_2 and α_3 chains (Heise et al., 1987). Alpha chain domains bind the β chain non-covalently, and together these form the class 1 MHC antigen. Alpha chain domains are membrane-spanning, while the β chain is entirely extracellular (Gill et al., 1987). Porcine β_2m genomic DNA was cloned by Singer et al. (1982).

Regulation

The β_2m gene is encoded on a different chromosome than is the heavy chain gene of the type I MHC (Flavell et al., 1986), and there are indications that β_2m expression in mouse endoderm (Jaffe et al., 1991), preimplantation human blastocyst (Jurisicova et al., 1996) and other cells (Morello et al., 1985; Israel et al., 1989), may be regulated independently from the genes encoding the α chain. Genes encoding both the light and heavy chains have NF-κB response elements in their regulatory regions (Israel et al., 989). Recently it has been demonstrated that β_2m expression can be upregulated by IL-6 in rat hepatocyte primary cell culture (Roncero et al., 1995).

Biological Effects

The major function of β_2m, as a component of class I MHC, is to help the immune system recognize cells altered by infection (Tarleton et al., 1992) or malignant changes (Hui, 1989). However, a soluble form of β_2m can be found in serum (Oh et al., 1975) and can affect gene expression by mimicking IGF-I function (Centrella et al., 1989). It can potentiate the expression of α chain class I MHC (Abdel Motal et al., 1993) and has proliferative actions on human bone-derived cells in vitro (Evans et al., 1991). Beta$_2$m is required for the immune response against intracellular parasites. For example, mice deficient in β_2m synthesis suffer high parasitaemias and early death when infected with the obligate cytoplasmic protozoan parasite *Trypanosoma cruzi* (Tarleton et al., 1992).

Role in Reproduction

Beta$_2$m mRNA is not detectable in human early preimplantation blastocysts (Desoye et al., 1988), but its expression is initiated shortly before implantation (Jurisicova et al., 1996). In the mouse, the developmentally-specific expression of β_2m is first detectable in the extra-embryonic derivatives of the early primitive streak stage embryo (Jaffe et al., 1990). In human (Jurisicova et al., 1996) and pig (Meziou et al., 1983) concepti, β_2m was first detected around the time of the implantation. It has been postulated that the regulation of MHC genes' expression during early development plays a critical role in maternal tolerance of the fetal allograft (Desoye et al., 1988). In addition, by mimicking the effect of IGF-I (Centrella et al., 1989), which induces steroidogenesis in porcine conceptuses (Green et al., 1995), β_2m may indirectly affect steroidogenesis.

CHAPTER 3
EXPRESSION OF LEUKEMIA INHIBITORY FACTOR AND INTERLEUKIN 6 GENES DURING PORCINE PREGNANCY

Introduction

Hematopoietic cytokines belong to the group of functionally (Piquet-Pellorce et al., 1994) and evolutionary (Shields et al., 1995) related cytokines that utilize a common receptor subunit, gp 130 (Hibi et al., 1990) to activate the Janus kinase (JAK)-signal transducer and STAT signaling pathway (Stahl et al., 1994). Leukemia inhibitory factor (LIF) and interleukin 6 (IL-6) are hematopoietic cytokines with several either known or proposed roles in pregnancies of diverse species (Hill, 1992; Mathialagan and Roberts, 1994).

LIF is believed to be involved in embryonic growth and development (Murray et al., 1990; Lavranos et al., 1995; Koshimizu et al., 1996), and suppression of differentiation of inner cell mass (ICM) (Williams et al., 1988; Hochereau de Reviers and Perreau, 1993; Savatier et al., 1996) and primitive ectoderm (Shen and Leder, 1992). LIF is essential for blastocyst implantation in the mouse (Stewart et al., 1992). Messenger RNA and / or protein of LIF has been detected in the human uterus (Shen and Leder, 1992), conceptus (Charnock-Jones et al., 1994) and placenta (Kojima et al., 1994) as well as in mouse uterus (Bhatt et al., 1991), conceptus and placenta (Conquet and Brulet, 1990). LIF is also expressed in rabbit (Yang et al., 1994), swine (Anegon et al., 1994) and cow (Brisson et al., 1996) uteri. LIF has been found to regulate the expression of other downstream target genes. Some of these genes may be important for reproduction, such as those regulating peptide (Ray et al., 1996; Nachtigall et al., 1996) or steroid (Stewart, 1994) hormone biosynthesis, and tissue remodeling (Harvey et al., 1995).

IL-6 is a physiologic regulator of expression of genes that have either documented or presumed role in establishment or maintenance of pregnancy (Croy et al., 1991; Stephanou and Handwerger, 1994; Yamaguchi et al., 1995). IL-6 mRNA and / or protein have been detected in mouse (Motro et al., 1990; De et al., 1992; De et al., 1993), human (Tabibzadeh et al., 1989, 1995), and swine uterus (Anegon et al., 1994), as well as in extraembryonic tissues of the mouse (Lee, 1992) and human (Stephanou et al., 1995). IL-6 mRNA was detected in days 13-21 pig conceptuses, as well as in periimplantation conceptuses of sheep and cows (Mathialagan et al., 1992).

Beta$_2$m is the light chain of the MHC complex, which is expressed in most healthy adult somatic cells (Governa and Biguzzi, 1976; Rosenthal et al., 1984). In early human embryos, expression of this gene is detectable at the blastocyst stage (Jurisicova et al., 1996), but in the mouse is only expressed in extraembryonic tissues, beginning at the primitive streak stage (Jaffe et al., 1990). In pig conceptuses, β_2m was visualized at the beginning of trophoblast elongation (Meziou et al., 1983).

Blastocyst implantation is a critical event in porcine reproduction, during which 30% of conceptuses do not survive (Pope, 1994). It was calculated that an increase in litter size would have the greatest impact on the economy of pig farming; greater than that which would result from any increases in rate of gain, lean / fat ratio, or obtaining a younger age at puberty (Tess et al., 1983). Signaling between the embryo and uterus is considered essential for implantation (Bazer et al., 1991). Revealing the role and timing of expression of signaling molecules and their receptors may help us better understand the processes leading to successful implantation and give a possible explanation as to why these cytokines are present in the uterine lumen during the periimplantation period. Cytokines are important signaling molecules in many organisms and in some cases, are essential for establishment of pregnancy (Stewart, 1994). Understanding the effects of cytokines on the porcine blastocyst may lead to development of strategies for improving the success rate of implantation, through genetic selection or biotechnology.

The objective of the following set of experiments was to directly compare LIF, IL-6 and β_2m mRNA expression in the domestic pig. It was important to conduct such a study, since many events in pregnancy are species-specific, and different regulatory molecules may have different roles among various species. Determination of the expression patterns of LIF, IL-6 and β_2m genes in porcine pregnancy will enable further investigations of the functions of these cytokines in this agriculturally important species. In this study, we examined whether these cytokines might play a role in reproduction of a species which has a non-invasive, epitheliochorial type of implantation. The hypotheses that LIF, IL-6 and β_2m genes are expressed in porcine endometrium, conceptuses and placenta during periimplantation and later pregnancy, and that β_2m has a constant level of expression, were examined.

Materials and Methods

Animals

Prepubertal crossbred gilts were obtained and mated in their second estrus. The day of onset of estrus was considered as day 0. Animals were slaughtered at days 0, 11, 12, 18, 30 and 60 of pregnancy at the Meats Processing Facility (University of Florida). Three animals were used for each day of pregnancy, except for day 11 of pregnancy, where six animals were used: three with spherical conceptuses and three with filamentous conceptuses found in their uteri. After exsanguination, the reproductive tracts were removed and immersed in ice. Uteri were transferred to a laminar flow hood where the uterine horns were trimmed from the mesometrium and blotted free of blood. Uteri of gilts, pregnant 18 days or less, were flushed with phosphate-buffered saline (PBS), pH 7.2. One half (3-6 conceptuses) of each day 11 litter was pooled for further analysis. Fetal membranes of day 18 conceptuses were dissected from embryos proper for further analysis. Uteri of gilts pregnant 30 days or more, were cut open and fetuses, together with fetal membranes, were detached from the uteri and chorioallantoic placentae were dissected. Endometria were dissected from myometria. All tissues were frozen at -80°C until later use.

Uterine cell cultures

Endometria of three day 12 pregnant gilts were taken by sterile technique to isolate uterine stromal (ST), luminal epithelial (LE) and glandular epithelial (GE) cells. For differential cell isolation, the method of Zhang et al. (1991) as modified by Reed et al. (1996a), was used. Luminal epithelial cells were isolated by digestion with dispase (Gibco) and pancreatin (Gibco) solution for 2.5 hours, and ST/GE by digestion with trypsin (0.04%), type II collagenase (0.06%) and DNase-I (0.01%), for 50 minutes at 37°C. ST cells were separated from the GE cells by passage of the cell suspension through a 38 μm stainless steel sieve (Thermos Scientific, Newark NJ). Stromal cells passed through the sieve with the filtrate, while glandular fragments were retained on the sieve. All three cell types were cultured in 100 mm tissue culture dishes (Corning Glassworks, Corning, NY) for about one week, at 37°C, in the atmosphere of 95% air and 5% CO_2. The total cellular RNA was isolated from cultures that reached 80-90% confluence.

Reverse transcription-polymerase chain reaction (RT-PCR)

RNA was isolated from 1 g of endometrium or placenta by the guanidinium thiocyanate method (Puissant and Houdebine, 1990). Conceptuses from the same litter and of the same morphology (spherical or filamentous) were pooled for RNA isolation. Total cellular RNA from conceptuses was isolated by adding 1 ml of TRIzol reagent (GIBCO BRL; Gaithersburg, MD) to the pool of conceptuses, followed by brief sonication, by the protocol provided with the reagent, except that the RNA was precipitated in isopropanol at -80°C overnight. Total cellular RNA was isolated from the endometrial primary cell cultures similarly as from conceptuses, except that 3 ml of TRIzol was used.

Complementary DNA was synthesized from 10 μg of total cellular RNA, using the cDNA Cycle kit (InvitroGen; San Diego, CA), following the protocol provided with the kit. RT was repeated once, as recommended for low molar mRNAs. The resulting first strand cDNA was phenol-extracted and ethanol precipitated in the presence of 1 μl of 2 mg/ml glycogen. After overnight precipitation, cDNA was recovered by centrifugation and dissolved in 100 μl of sterile distilled water.

PCR primers for amplification of LIF cDNA were designed from the porcine genomic LIF sequence (Willson et al., 1992). PCR primers for detecting porcine IL-6 cDNA were designed by Mathialagan et al. (1992) and for detection of porcine β_2m cDNA by Reddy et al. (1996), based on the sequence by Milland et al. (1993). The primer sequences, expected PCR product sizes, locations with regard to exons and positions on the cDNA sequence, are shown in Table 3-1. As the porcine genomic sequence of IL-6 is not available, exons to which these PCR primers anneal are not known. However, for IL-6 primers, it was possible to infer the genomic organization based on the human (Gough et al., 1988) and mouse (Tanabe et al., 1988) IL-6 genomic sequences. Oligonucleotide primers were synthesized by the DNA Synthesis core of the Interdisciplinary Center for Biotechnology Research at the University of Florida (Gainesville, FL).

Thermostable *Taq* DNA polymerase (Boehringer Mannheim, 5 units per μl) was used in the PCR, in the amount of 1 unit per reaction. Two out of 100 μl of cDNA from day 30 porcine placentae were used for optimization of IL-6 and β_2m cDNA PCR conditions, particularly regarding the optimal pH and Mg^{2+} concentration, by use of the PCR Optimizer Kit (Invitrogen; San Diego, CA). Twenty out of 100 μl cDNA from day 30 porcine placentae were used for optimization of LIF PCR conditions. Concentrations of Mg^{2+} ranged from 1.5 to 3.5 mM, while the pH varied between 8.5 and 10.0. Buffer “J” (5x) was used in subsequent amplifications of the IL-6 cDNA, providing 2.0 mM Mg^{2+}, 50

Table 3-1. Sequences of PCR primers used in the study.

Gene	Sequence	Product size	Exon	Position	Species	Reference
β_2 m	5’ CTGCTCTCACTGTCTGG 3’ 5’ ATCGAGAGTCACGTGCT 3’	287 bp	1[a] 2[a]	91 378	pig	Milland et al., 1993
LIF	5’ CCATGTCACAGCAACCTCATGAACC 3’ 5’ TATACTTCCCCAGGAGCTGACAGCC 3’	459 bp	2 3	115 549	pig	Willson et al., 1992
IL-6	5’ GACGGATGCTTCCAATCTG 3’ 5’ TCCAAGAAACAACCTGGC 3’	654 bp	3[b] 5[b]	357 993	pig	Mathialagan et al., 1992

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[a] inferred on the basis of the human gene structure (Gussow et al., 1987)

[b] inferred on the basis of the human (Gough et al., 1988) and mouse (Tanabe et al., 1988) gene structures

mM KCl, pH 9.5, in a reaction volume of 50 µl. PCR buffer (10x) provided with the *Taq* DNA polymerase by Boehringer Mannheim, was used for amplification of LIF and β_2m cDNAs. The conditions provided by this buffer were: 1.5 mM Mg^{2+}, 50 mM KCl, pH 8.3. In addition, each template-primer combination was validated for the number of thermocycles used and the starting template concentration to ensure that synthesis remained in the exponential phase. The cycling parameters were the following: 94°C for 1 minute and 30 seconds; 55°C for 2 minutes and 72°C for 2 minutes. The number of cycles was 25 for β_2m, 35 for IL-6 and 40 for LIF cDNAs. After completion of all cycles, an additional 10 minutes of extension at 72°C was performed. Once optimal conditions were defined, all samples for a given experiment were simultaneously amplified and analyzed. RT-PCR products were electrophoresed in 1.5% agarose gels, and photographed when illuminated with ultraviolet (UV) light. The molecular weight markers were λ/HinfI and pUC18/Sau$_3$AI. The products were transferred from gels to Biotrans nylon membrane (ICN Biochemicals; Aurora, OH), and cross linked by UV light for 2 minutes. DNA probes were radiolabeled using a Nick Translation kit (Amersham Life Sciences; Arlington Heights, IL). About 200 ng of LIF, IL-6 and β_2m cDNAs were radiolabeled with 50 µCi of ^{32}P αdCTP (3,000 Ci/mmol) and purified from unincorporated radioisotope by gel filtration chromatography on Sephadex G-50 columns (Pharmacia, Piscataway, NJ). Southern hybridization was performed by the method in Maniatis et al. (1982) at 65°C overnight and membranes exposed to X-ray film at -20°C overnight.

PCR products analyzed by gel electrophoresis and Southern-hybridization, were visually evaluated by one more person in addition to the author. The band intensity was described as: very intense (+++), intense (++), visible (+), trace - visible only by Southern hybridization (+/-) and not visible (-).

DNA cloning and sequencing

Fragments of LIF, IL-6 and β_2m cDNA were cloned into the *pCR II* TA cloning vector (Invitrogen; San Diego, CA). Ligations were used in transformation of *E. coli* One Shot (Invitrogen; San Diego, CA). Plasmid DNA was isolated using the QIAprep-spin plasmid isolation kit (Qiagen; Chatsworth, CA). Plasmid DNAs were sequenced using the Sequenase-based DNA sequencing kit by United States Biochemicals (Cleveland. OH). Specific PCR primers were used in the sequencing reactions. All sequence information was analyzed using the Sequence Analysis Software package from the Genetics Computer Group (Madison, WI).

Results

Optimization and Validation of PCR conditions

Complementary DNA from day 60 placenta was used in PCR (IL-6 and β_2m primer sets) under different conditions and products analyzed in 1.5% agarose gels. Mg^{2+} concentrations were varied from 1.5 to 3.5 mM, and pH was varied between 8.5 and 10.0 in attempts to optimize PCR efficiency and fidelity. Products of a similar size resulted from all IL-6 PCRs, with the reaction buffer “J” (Invitrogen) yielding the most product. Buffer “J” established a final concentration of 2.0 mM Mg^{2+} and final pH of 9.5. For β_2m PCR, there was a similar amount of product with all tested Invitrogen buffers and one

Boehringer Mannheim PCR buffer. The Boehringer Mannheim buffer (pH 8.3 and 0.15 mM $MgCl_2$) was used for all subsequent amplifications of β_2m cDNA.

Figure 3-1 shows accumulation of porcine cDNA in successive cycles of PCR (15 to 50), as monitored by electrophoresis in a 1.5% agarose gel. In the PCR for porcine LIF, the amount of product obtained with 40 thermocycles was judged to be in the exponential phase of PCR. Therefore, PCR with 40 cycles was chosen for subsequent amplifications of LIF cDNA. In PCRs for IL-6 and β_2m cDNAs, the amounts of products obtained with 35 and 25 cycles, respectively, were judged to be in the exponential phases, and these cycle numbers were used in all subsequent reactions.

Conceptus Expression of LIF, IL-6 and β_2m mRNAs

Photographs of agarose gels and autoradiograms of southern blots containing RT-PCR products from day 11 porcine conceptuses, for LIF, IL-6 and β_2m mRNAs, are shown in figure 3-2. No LIF RT-PCR product from spherical or filamentous day 11 conceptuses was visible when electrophoresed on agarose gel. In day 11 spherical conceptuses, LIF was detected only by Southern hybridization of RT-PCR products. IL-6 was expressed exclusively in day 11 filamentous, but not in day 11 spherical conceptuses. Beta$_2$m was expressed in day 11 litters of both morphologies, with some variation in intensity evident, regardless of the morphology.

Placental Expression of LIF, IL-6 and β_2m mRNAs

Figure 3-3 depicts photographs of agarose gels and autoradiograms of Southern blots for LIF, IL-6 and β_2m RT-PCR products obtained from total cellular RNA of days 18, 30 and 60 porcine placentae. LIF cDNA was observed on agarose gels in days 30 and

60, but not day 18, placentae. In day 18 placentae, LIF mRNA was detected only by Southern hybridization of PCR products. IL-6 mRNA was expressed in day 18, but more abundantly in days 30 and 60 placentae. Beta$_2$m products were consistently obtained from all placental samples examined.

Endometrial Expression of LIF, IL-6 and β_2m mRNAs

Figure 3-4 shows photographs of agarose gels and autoradiograms of Southern blots containing RT-PCR products from days 0, 11, 18, 30 and 60 porcine embryos, for LIF, IL-6 and β_2m mRNAs. LIF mRNA was detected sporadically in day 11 endometria bearing spherical conceptuses, with the most abundant RT-PCR product from day 30 endometria. In day 0 endometria, LIF mRNA was detected only by Southern hybridization of PCR products. IL-6 mRNA was amplified from day 0 and day 11 endometria with conceptuses of both morphologies examined, but more abundantly from day 30 and 60 endometria. This mRNA was not detected in day 18 endometrial tissues. Beta$_2$m RT-PCR products were evenly distributed across all endometrial samples examined.

Endometrial Cell Expression of LIF, IL-6 and β_2m mRNAs

Figure 3-5 shows photographs of agarose gels containing RT-PCR products from day 12 porcine endometrial cells (ST, LE and GE), amplified using primers for LIF, IL-6 and β_2m. LIF was not detected in any of endometrial cell types. IL-6 mRNA was amplified in all cell types, but was more abundant in ST and GE than LE cells. Beta$_2$m PCR products were consistent in yield across all endometrial cell types examined.

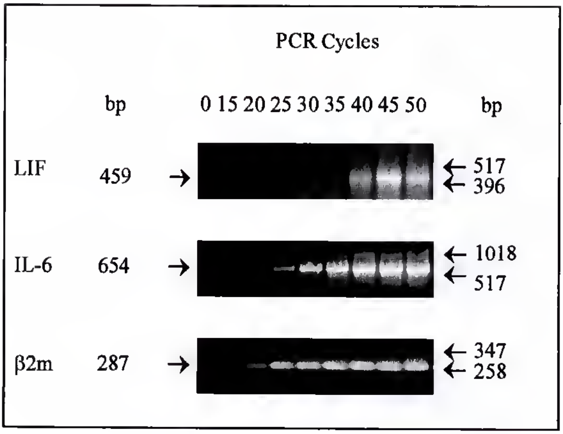

Figure 3-1. Ethidium bromide-stained agarose gels containing RT-PCR products amplified from day 60 placenta for 15 to 50 cycles, using primers for LIF, IL-6 and β_2m.

The numbers on the left are expected PCR product sizes. The numbers on the right are sizes of DNA markers (λ/HinfI for LIF and IL-6 and pUC18/Sau_3AI for β_2m).

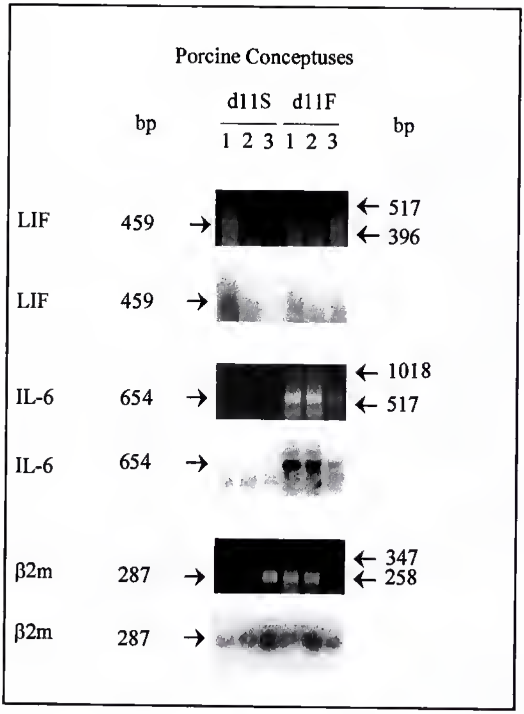

Figure 3-2. Ethidium bromide-stained agarose gels containing pools of day 11 conceptus cDNAs, amplified by PCR using primers for LIF, IL-6 and β_2m. Southern blots of PCR products were hybridized with ^{32}P labeled probes for LIF, IL-6 and β_2m, respectively (lower parts for each gene).

The numbers of PCR cycles for obtaining LIF, IL-6 and β_2m products were 40, 35 and 25, respectively. The numbers on the right are sizes of DNA markers (λ/HinfI for LIF and IL-6 and pUC18/Sau$_3$AI for β_2m); d11S = day 11 spherical conceptuses; d11F = day 11 filamentous conceptuses; 1, 2 and 3 are different pools of conceptuses from each morphology group.

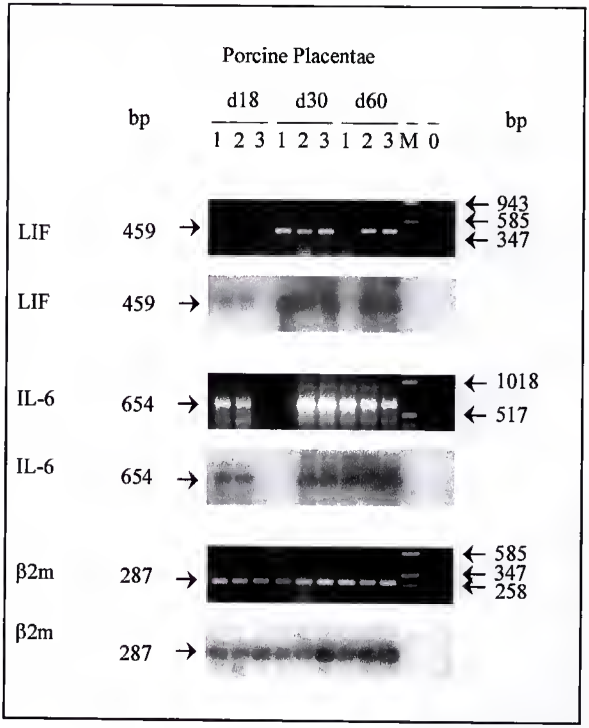

Figure 3-3. Ethidium bromide-stained agarose gels containing days 18, 30 and 60 placental cDNAs, amplified by PCR using primers for LIF, IL-6 and β_2m. Southern blots of PCR products were hybridized with ^{32}P labeled probes for LIF, IL-6 and β_2m, respectively (lower parts for each gene).

The numbers of PCR cycles for obtaining LIF, IL-6 and β_2m products were 40, 35 and 25, respectively. The numbers on the left are expected PCR product sizes. The numbers on the right are sizes of DNA markers (λ/HinfI for IL-6 and pUC18/Sau$_3$AI for LIF and β_2m); d18 = day 18 placenta; d30 = day 30 placenta; d60 = day 60 placenta; M = DNA marker; 0 = no template).; 1, 2 and 3 are different animals.

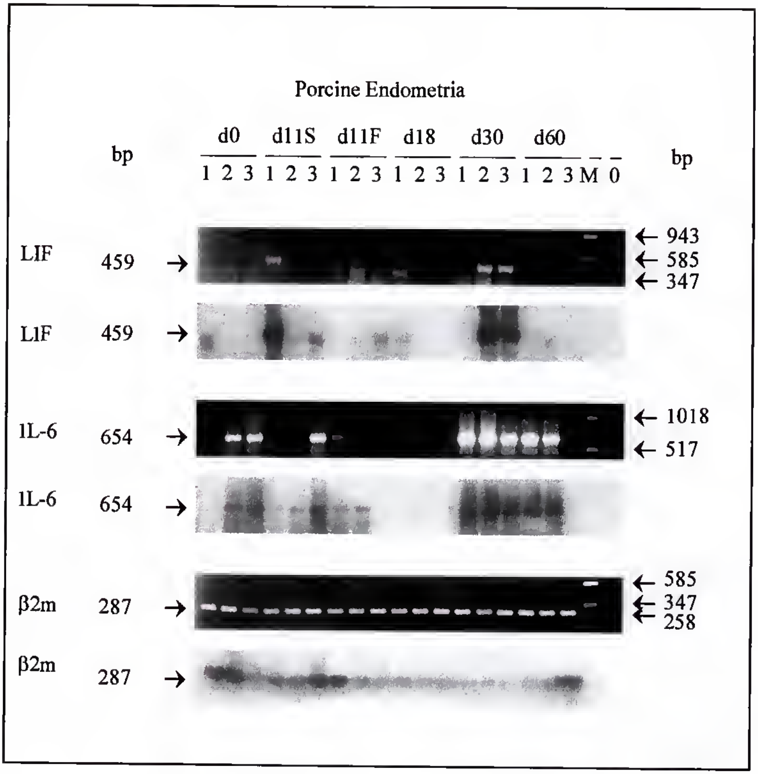

Figure 3-4. Ethidium bromide-stained agarose gels containing days 0, 11, 18, 30 and 60 endometrial cDNAs, amplified by PCR using primers for LIF, IL-6 and β_2m. Southern blots of PCR products were hybridized with ^{32}P labeled probes for LIF, IL-6 and β_2m, respectively (lower parts for each gene).
The numbers of PCR cycles for obtaining LIF, IL-6 and β_2m products were 40, 35 and 25, respectively. The numbers on the left are expected PCR product sizes. The numbers on the right are sizes of DNA markers (λ/HinfI for IL-6 and pUC18/Sau3AI for LIF and β_2m); d0 = day 0; day11S = day 11 endometrium bearing spherical conceptuses; day11F = day 11 endometrium bearing filamentous conceptuses; d18 = day 18 endometrium; d30 = day 30 endometrium; d60 = day 60 endometrium; M = DNA marker; 0 = no template).; 1, 2 and 3 are different animals.

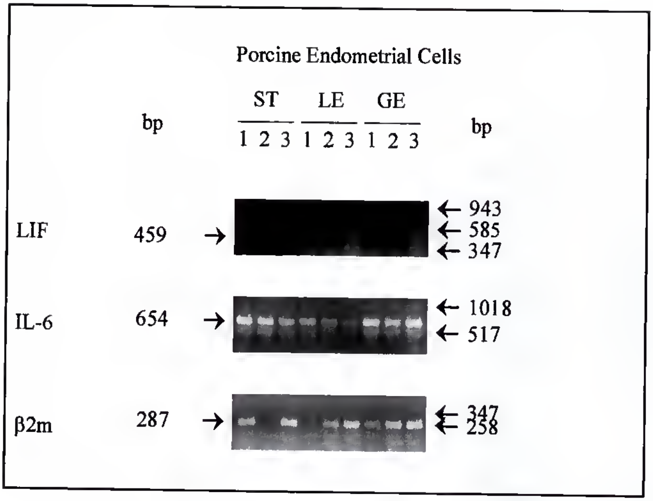

Figure 3-5. Ethidium bromide-stained agarose gels containing day 12 endometrial primary cell culture cDNAs, amplified by PCR using primers for LIF, IL-6 and β_2m.

The numbers of PCR cycles for obtaining LIF, IL-6 and β_2m products were 40, 35 and 25, respectively. The numbers on the right are sizes of DNA markers (λ/HinfI for IL-6 and pUC18/Sau$_3$AI for LIF and β_2m); ST = stromal cells, LE = luminal epithelial, GE = glandular epithelial cells; 1, 2 and 3 are cells from different animals.

Table 3-2. Summary of RT-PCR amplification of LIF, IL-6 and β_2m mRNA in porcine conceptuses, placentae, endometrium, and endometrial cell cultures.

Gene	**Conceptus**		**Placenta**			**Endometrium**						**Endometrial cells**		
	d11S	d11F	d18	d30	d60	d0	d11S	d11F	d18	d30	d60	ST	LE	GE
LIF	+/-	-	+/-	++	++	+/-	+	+/-	-/+	+++	-	-	-	-
IL-6	-	++	++	+++	+++	++	+	+	-	+++	+++	++	+	++
β_2m	++	++	++	++	++	++	++	++	++	++	++	++	++	++

Tissues from three animals were analyzed by PCR hybridization and evaluated visually. The band intensity was described as: very intense (+++), intense (++), visible (+), trace - visible only by Southern hybridization (+/-) and not visible (-).

PCR Summary

The results of this survey for LIF, IL-6 and β_2m mRNA expression in porcine conceptuses, placentae, endometria and endometrial cell lines, are summarized in Table 3-2.

Discussion

RT-PCR is a method limited by the fact that the amplification of DNA is exponential, therefore a little variations in initial amount of mRNA between samples can result in great differences in the final product (Dukas et al., 1993). To prevent large variations of product resulting from the same amount of mRNA, positive and negative controls were used. Negative PCR control tube contained all components as the sample mixtures except template cDNA. Primers for β_2m were used to amplify positive controls. Sometimes, variations in the amount of PCR products between animals were observed. It is not known for each particular sample whether the source of the variation was the method or biological variations between the animals, such as developmental stage, number of conceptuses, endocrine profiles or feto-placental weights. Another limitation of RT-PCR is the fact that this method detects mRNA, and not protein, the final product of gene expression. In some instances, a major regulation of gene expression takes place after mRNA has been synthesized (Raqib, 1996).

LIF mRNA expression was detected in endometrium at the stage immediately preceding implantation. These findings are comparable to detection of LIF at similar stages in the human (Shen and Leder, 1992), mouse (Bhatt et al., 1991), rabbit (Yang et al., 1994), cow (Brisson et al., 1996) and swine (Anegon et al., 1994) preimplantation endometrium. However, the amount of detected LIF mRNA in pig preimplantation endometrium was very low, compared to LIF mRNA detected in day 30 endometrium, and days 30 and 60 placentae. LIF mRNA was undetectable in day 11 conceptuses, in contrast to the reports on LIF expression in human (Charnock-Jones et al., 1994) and mouse (Conquet and Brulet, 1990) preimplantation conceptuses.

Expression of LIF mRNA was detected in days 30 and 60 placentae and day 30 endometrium. These findings are similar to the reports from a mouse study, in which LIF transcripts were detected in preimplantation blastocysts and continued to be expressed in the extraembryonic tissues up to midgestation (Conquet and Brulet, 1990). In human endometrium, LIF expression increases at the time of implantation, while it is low in the first trimester and term placenta (Kojima et al., 1994).

The function of LIF in the endometrium and placenta after implantation is unknown. It may possibly regulate the expression of genes that are expressed in the later stages of pregnancy, such as ALP, TIMPs 1-3, MMP, uPA, cytochrome $P450_{arom}$, GHRF and hCG. These gene products are synthesized and/or secreted in reproductive tracts of various species. They have proposed or established roles in pregnancy, and may be regulated by LIF in either a positive or negative manner. Some of these genes are proteinase and proteinase inhibitors, involved in tissue remodeling processes, while others are involved in sex hormone synthesis. Proteinases MMP-9 and uPA have been detected in the mouse trophoblast, and their synthesis is suppressed by LIF (Harvey et al., 1995). Proteinase inhibitor, ALP is expressed in porcine, ovine and bovine endometrium (Badinga et al., 1994) and was also found to be positively modulated by LIF (Chapter 5). Proteinase inhibitors, TIMPs 1-3 are expressed in the mouse uterus (Waterhouse et al., 1993;

Graham et al., 1994) and in bovine oviducts and granulosa cells (Satoh et al., 1994). In early mouse embryos, genes encoding proteins from the TIMP group were induced by LIF (Harvey et al., 1995), while in lung fibroblasts both LIF and IL-6 stimulated the expression of TIMP-1 mRNA (Richards et al., 1993). Steroidogenic enzyme, cytochrome $P450_{arom}$ is expressed in the porcine placenta (Choi et al., 1996) and is induced by JAK/STAT pathway in human adipose tissue (Zhao et al., 1995). The peptide hormone, GHRF, is secreted by cultured mouse placental cells and is inhibited by LIF (Yamaguchi et al., 1995). The peptide hormone, chorionic gonadotropin, is synthesized in the primate and equine, but not porcine placenta or uterus (King, 1993). In the human choriocarcinoma cell line (Kojima et al., 1995) and cytotrophoblast from term placentae (Nachtigall, et al., 1996), hCG synthesis is suppressed by LIF.

The differences in the course of pregnancy between animal species may be correlated with LIF expression. The mouse and rabbit exhibit endotheliochorial placentation, while humans have the hemochorial type of placentation. Both types of placentation are invasive and characterized by decidualization (Mossman, 1987). Horse, cattle, swine and other ungulates exhibit epitheliochorial placentation. In these species, the type of placentation is much less invasive, and the decidua is absent (Mossman, 1987). No study has been performed to directly compare the levels of LIF expression between these species, but it appears that the level of LIF expression in pig and cow at the time of implantation is lower than in the mouse and human endometrium. It is possible that LIF has a different function in species that exhibit endotheliochorial or hemochorial placentation versus epitheliochorial placentation type. This is in agreement with findings by Bischof et al. (1995), who postulated that LIF inhibits the differentiation of human cytotrophoblastic cells (CTB) towards an invasive phenotype by inhibiting the secretion of gelatinase. While inhibiting the secretion of proteinases, LIF may also regulate the invasiveness of trophoblast by stimulating the expression of metalloproteinase inhibitors (Richards et al., 1993; Harvey et al., 1995).

The differences among species in LIF synthesis may be ascribed to the differences in the levels of estrogen present in the ULF of various species. Porcine conceptuses synthesize large amounts of estrogen (Bazer and Thatcher, 1977). However, it is not clear what role, if any, estrogen plays in the regulation of LIF expression. In the rhesus monkey, LIF cDNA was detected in progesterone (P_4), but not E_2-dominated uterus (Ace and Okulicz, 1995), suggesting the possible inhibitory effect of E_2 on LIF expression. There is no direct evidence that steroid hormones (E_2 and progestins) regulate LIF mRNA or protein synthesis by human endometrial cells in culture (Arici et al., 1995). From the data presented, it appears that in pig LIF may have a greater role during the later stages of pregnancy, than during the implantation phase.

No LIF mRNA was detected in cultured endometrial cells. The reason for that may be that each type of cells was cultured separately from the other types and conceptuses, lacking potential stimulatory signals that may be produced by these cells. LIF expression has been detected in mouse (Bhatt et al,. 1991) and rabbit (Yang et al., 1994) glandular endometrial epithelium as well as in human glandular (Charnock-Jones et al., 1994; Arici et al., 1995; Chen et al., 1995; Cullinan et al., 1996) epithelium, and in lesser extent, in endometrial stroma (Chen et al., 1995).

Expression of IL-6 mRNA is detectable in endometrium on day 0, 11, 30 and d60, and in the placenta on days 18, 30 and 60. The highest levels of IL-6 mRNA in both tissues were detected on days 30 and 60 of pregnancy. IL-6 mRNA expression in day 11 conceptuses is strikingly different between the spherical and filamentous conceptuses, which transform from one morphology to another over several hours (Geisert et al., 1982). This observation may indicate that induction of IL-6 gene expression is coupled to the morphological transformation of conceptuses. A study of differential gene expression between these two stages was done by Green et al. (1995). They found a sharp decline in the expression of CYP19 and CYP17, at the time when the conceptus morphology changes from spherical to filamentous. Since these genes encode cytochromes $P450_{arom}$ and P450c17, the enzymes catalyzing the sex steroid synthesis (Jefcoate, 1986; Miller, 1988; Hanukoglu, 1992), and estrogen is a suppresser of the IL-6 promoter (Ray et al., 1994; Stein and Yang, 1995), it is possible that estrogen suppresses IL-6 gene expression in spherical embryos. Since the estrogen effect on the porcine endometrium is transient (Simmen et al., 1990), the IL-6 gene may be de-repressed soon after the aromatase synthesis by the conceptus ceases. Undetectable levels of IL-6 expression in day 18 endometria, when the second peak of estrogen synthesis by the conceptus was detected (Ko et al., 1994), are in line with this model. IL-6 was expressed in day 18, but more abundantly in days 30 and 60 placentae. Although estrogen, a possible IL-6 suppressor, is synthesized by day 18 porcine conceptuses (Ko et al., 1994), as well as by day 30 and 60 porcine placenta and endometrium (Choi et al., 1996), its levels may not be sufficient to totally suppress IL-6 gene expression.

A possible function of IL-6 during pregnancy is in the regulation of plasminogen activators, which are stimulated by IL-6 (Samad et al., 1994), and have a proposed role in implantation (Graham and Lala, 1992). IL-6 also stimulates expression of genes from the

TIMP group (Sato et al., 1990), which influence the activation of the prometalloproteinase and act to modulate proteolysis of extracellular matrix (Denhardt et al., 1993), which takes place during mouse implantation (Sappino et al., 1989). IL-6 has been demonstrated to prevent apoptosis in early plasma cells (Kawano et al., 1995), thymocytes (Kuida et al., 1995) and prevent TGF-β-1-induced apoptosis in M1 myeloid leukemia cells (Lotem and Sachs, 1992). In other instances, IL-6 induced apoptosis in M1 myeloid cell line (Minami et al., 1996) and in murine hematopoietic cell line, Y6 (Oritani et al., 1992). Apoptosis, or programmed cell death, has been linked to reproductive processes, such as endometrial involution in the mouse (Murdoch, 1995) blastocyst implantation in the mouse (Alexander et al., 1996) and embryonic development in *Drosophila melanogaster* (Pronk et al., 1996). Another possible role of IL-6 in the porcine reproductive tract is to enhance antibody presentation by epithelial and stromal cells of the uterus (Prabhala and Wira, 1995), which may be important in resistance to reproductive tract infections. It can also stimulate synthesis of immunoglobulins of the A (Ramsay et al., 1994) and E (Vercelli et al., 1989) class.

In summary, the expression of IL-6 by the conceptus at the time of implantation may indicate a role in implantation. The fact that both LIF and IL-6 are more highly expressed by both the endometrium and placenta in later stages of pregnancy, indicates their possible roles in feto-maternal communication.

Beta$_2$m PCR products were evenly distributed across fresh placental and endometrial tissue samples, as well as in the endometrial cell cultures. These findings are in agreement with findings from analysis of somatic cells of various species (Governa and Biguzzi, 1976; Klein and O'hUigin, 1993). The relative amounts of β_2m RT PCR products were variable between different litters of day 11 embryos, with the amount of product in filamentous embryos being slightly higher. This may be a result of the beginning of expression of β_2m in porcine embryos, when not all embryos express β_2m to their maximal levels. This would be in agreement with immunocytochemical localization of β_2m in elongating, but not spherical, porcine conceptuses (Meziou et al., 1983). In human and mouse embryos, β_2m expression also appears to start around the time of the implantation; in human at the blastocyst stage (Jurisicova et al., 1996), while in the mouse at the primitive streak phase (Jaffe et al., 1990). This study showed increased IL-6 expression when the conceptuses changed morphology from spherical to filamentous. Since IL-6 induces β_2m expression in rat hepatocytes primary cell culture (Roncero et al., 1995) it may have similar effect on porcine embryos.

CHAPTER 4

EXPRESSION OF LEUKEMIA INHIBITORY FACTOR RECEPTOR BETA GENE DURING PORCINE PREGNANCY: ISOLATION AND CHARACTERIZATION OF A COMPLEMENTARY DNA FRAGMENT

Introduction

LIF is essential for blastocyst implantation in the mouse (Stewart et al., 1992), and has several proposed roles during pregnancy of various species (Murray et al., 1990; Shen and Leder, 1992; Hochereau de Reviers and Perreau, 1993; Kojima et al., 1994; Yang et al., 1994; Lavranos et al., 1995; Koshimizu et al., 1996; Savatier et al., 1996). LIF mRNA and protein have been detected in female reproductive tract tissues of several species. This gene is expressed in the periimplantation mouse (Bhatt et al., 1991), human (Kojima et al., 1994; Charnock-Jones et al., 1994), rabbit (Yang et al., 1994), cow (Brisson et al., 1996) and pig (Anegon et al., 1994) endometrium at the time of implantation. The LIF gene is also expressed in mouse preimplantation blastocysts and extraembryonic tissues (Conquet and Brulet, 1990) as well as in mouse (Conquet and Brulet, 1990) and human (Kojima et al., 1994) placenta.

LIF stimulates the expression of adrenocorticotropic hormone (ACTH) and POMC in human pituitary cells in culture (Akita et al., 1995), by inducing the STAT signal transduction pathway (Ray et al., 1996). This cytokine also stimulates TIMP-1 at the mRNA level in lung fibroblasts of synovial origin (Richards et al., 1993). LIF inhibited the mRNA expression of uPA and MMP-9 in mouse blastocysts (Harvey et al., 1995) as well as hCG at the mRNA and protein levels (Nachtigall et al., 1996) in cultures of human cytotrophoblasts. All of these molecules have established or proposed roles in pregnancy.

Like other functionally (Rose and Bruce, 1991; Piquet-Pellorce et al., 1994) and evolutionarily (Shields et al., 1995) related cytokines of the hematopoietic cytokine group, LIF utilizes as a receptor subunit, gp 130 (Hibi et al., 1990), a characteristic for all members of this group (Wijdenes et al., 1995; Minami et al., 1996). Subunit gp130 is the signaling molecule that activates the JAK-TYK family of cytoplasmic tyrosine kinases (Stahl et al., 1994). In order to activate the JAK-TYK enzymes, LIF binds to LIFR-β, which in turn, forms a heterodimer with gp130, leading to phosphorylation of JAK-TYK (Stahl et al., 1994). Phosphorylated protein-tyrosine kinases activated by receptor-kinase complexes, recruit members of STAT family and in turn activate these by phosphorylation. As a consequence, the phosphorylated STAT proteins dimerize, translocate into the nucleus, where they bind specific response elements in the promoters of target genes and stimulate their transcription. Gene modulation events involving JAK-TYK and STAT have been dubbed the JAK-STAT pathway (Heim, 1996).

LIFR-β is a cell surface receptor. Like other hematopoietic cytokine receptors, LIFR-β belongs to a large superfamily of transmembrane proteins, characterized by a 200 amino acid extracellular sequence containing the ligand binding domain. The gene encoding LIFR-β has been mapped to human chromosome 5, where it is linked to the genes for the receptors for IL-3 and GM-CSF (Baird et al., 1995). LIFR-β is present in normal mouse serum, in a truncated form, as a soluble receptor or LIF binding protein (LBP). The concentrations of LBP are elevated in pregnant mouse serum (Layton et al., 1992), where it may serve as an inhibitor of the systemic effects of locally produced LIF.

LIFR-β expression has been detected in the mouse conceptus (Yoshida et al., 1994) and endometrium (De et al., 1992), as well as in human blastocysts (Charnock-Jones et al., 1994), endometrium (Cullinan et al., 1996) and placenta (Kojima et al., 1995). Indirect evidence that LIFR-β mRNA is synthesized by the porcine conceptus is the observation that LIF inhibits the differentiation of cultured pig embryonic disc cells *in vitro* (Hochereau de Reviers and Perreau, 1993). However, it is not known whether porcine trophoblast can be a target tissue for LIF. Besides LIF, LIFR-β is utilized by CNTF (Davis et al., 1993) and by OSM (Rose et al., 1994). Specific antibodies to distinct regions of gp130 were developed, that inhibit binding of single cytokines of the hematopoietic group to gp130 (Wijdenes et al., 1995; Chevalier et al., 1996).

It is not known how the gene encoding LIFR-β is regulated. Human and mouse LIFR-β genes have two conserved sequences acting as transcriptional start sites. The first is 60-64 base pairs upstream of the translational start codon and immediately downstream of a TATA box. The second transcriptional start site is 160 base pairs upstream of the start codon and adjacent to a "TATA-like" element (Stahl et al., 1990).

Expression of LIFR-β in the porcine reproductive tract or conceptus has not been previously reported. It is not known whether LIF plays an important role in porcine pregnancy. Detecting LIFR-β in porcine embryonic tissues would suggest that LIF, or some other cytokine acting via LIFR-β, may be a differentiation inhibiting factor in the porcine blastocyst. Detection of LIFR-β in porcine endometrium may suggest a role in implantation-regulating events. It is important to investigate a pattern of LIFR-β gene expression, since many events in pregnancy are species-specific (Bazer et al., 1991), and different molecules may have different roles across various species. Once the expression pattern of LIF in porcine pregnancy is known, it will be possible to speculate, and further investigate, its function.

In this study, we examined whether LIF might also play a role in reproduction of a species which has a non-invasive, epitheliochorial, type of implantation. This study tested the hypothesis that LIFR-β is expressed in porcine conceptuses, placenta and endometrium, during the periimplantation period, and later in pregnancy. The hypothesis was tested by designing the original LIFR-β PCR primers, based on mouse and human sequences, for RT-PCR studies and examining for the expression of mRNA in porcine blastocyst, placenta and endometrium.

Materials and Methods

Animals and Uterine cell cultures

Animals were treated as described in the Chapter-3. Uterine cells were isolated and cultured as described in Chapter-3.

Reverse-transcription-Polymerase Chain Reaction

Total cellular RNA was isolated and reverse-transcribed as described in Chapter-3. PCR primers for amplification of LIFR-β cDNA were designed based on conserved regions between the human (Gearing et al., 1991) and mouse (Tomida et al., 1993) cDNA sequences. Highly conserved sequences were chosen for primer design. The primer sequences, expected PCR product sizes, locations with regard to exons and positions on the cDNA sequence, are shown in Table 4-1. Genomic organization of porcine LIFR-β is not available for determining to which exons LIFR-β PCR primers have affinity to anneal. Oligonucleotide primers were synthesized by the DNA Synthesis core of the Interdisciplinary Center for Biotechnology Research at the University of Florida (Gainesville, FL).

Thermostable *Taq* DNA polymerase (Boehringer Mannheim, 5 units per µl) was used in PCR (1 unit per reaction). Ten out of 100 µl of cDNA from day 30 porcine placenta were used for optimization of LIFR-β cDNA PCR conditions, particularly as regards optimal pH and Mg^{2+} concentrations, using the PCR Optimizer Kit (Invitrogen; San Diego, CA). Concentrations of Mg^{2+} ranged from 1.5 to 3.5 mM, while the pH was varied from 8.5 to 10.0. Buffer "J" (5x) was used in subsequent amplifications of the LIFR-β cDNA. Final conditions were 2.0 mM Mg^{2+}, 50 mM KCl, pH 9.5, in a reaction volume of 50 µl. In addition, the template-primer combination was validated for optimal number of thermocycles and starting template concentration to ensure that synthesis of final product was in the exponential phase. The cycling parameters were the following: 94°C for 1 minute and 30 seconds; 55°C for 2 minutes and 72°C for 2 minutes. The number of cycles was 40. After completion of all of these cycles, an additional 10 minutes of extension at 72°C was performed. Once optimal conditions were defined, all samples for a given experiment were simultaneously amplified and analyzed.

RT-PCR products were electrophoresed in 1.5% agarose gels, and photographed when illuminated with ultraviolet (UV) light. The molecular weight markers were λ/HinfI

Table 4-1. Sequences of PCR primers used in this study.

Gene	Sequence	Product size	Position[a]	Species	Reference
LIFR-β	5’ GGAAGCCTTTACCCATTAATGAAGC 3’ 5’ TGTTTGAGGGAACNTTTCTCCAGTC 3’	375 bp	1845 2195	human / mouse	Gearing et al., 1991; Tomida et al., 1993

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[a] human cDNA sequence.

and pUC18/Sau$_3$AI. The PCR products were transferred from gels to Biotrans nylon membranes (ICN Biochemicals; Aurora, OH), and cross linked by UV light for 2 minutes. DNA probes were radiolabeled using a Nick Translation kit (Amersham Life Sciences; Arlington Heights, IL). About 200 ng of LIFR-β cDNAs were radiolabeled with 50 μCi of ^{32}P αdCTP (3,000 Ci/mmol) and purified from unincorporated radioisotope by gel filtration chromatography on Sephadex G-50 columns (Pharmacia, Piscataway, NJ). Southern hybridization analysis was performed by the method in Maniatis et al. (1982) using the temperature of 65°C overnight and membranes exposed to X-ray film at -20°C overnight.

PCR products analyzed by gel electrophoresis and Southern-hybridization, were visually evaluated by one more person in addition to the author. The band intensity was described as: very intensive (+++), intensive (++), visible (+), traces - visible only by Southern hybridization (+/-) and not visible (-).

DNA cloning and sequencing

LIFR-β cDNA fragments were cloned using the *pCR II* TA cloning vector (Invitrogen; San Diego, CA). Ligations were used in transformation of *E. coli* One Shot (Invitrogen; San Diego, CA). Plasmid DNA was isolated using the QIAprep-spin plasmid isolation kit (Qiagen; Chatsworth, CA). Plasmid DNAs were sequenced using the Sequenase-based DNA sequencing kit (United States Biochemicals; Cleveland, OH). Specific LIFR-β PCR primers were used in the sequencing reactions. All sequence information was analyzed using the Sequence Analysis Software package from the Genetics Computer Group (Madison, WI).

Results

Optimization of PCR Conditions

Complementary DNA from day 60 porcine placenta was used as a template in PCR under different conditions using the LIFR-β primer set and products analyzed in 1.5% agarose gels. Mg^{2+} concentrations were varied from 1.5 to 3.5 mM, and pH from 8.5 to 10.0. Products of a similar size resulted from all PCRs using LIFR-β primers, with the reaction using buffer "J" (Invitrogen) resulting in the most PCR product. The final conditions with buffer "J" were 2.0 mM Mg^{2+} and pH 9.5.

Figure 3-1 shows accumulation of porcine cDNA in successive cycles of PCR (15 to 50), electrophoresed in a 1.5% agarose gel. In PCR using primers for porcine LIFR-β, the amount of product obtained after 40 thermocycles was judged to be in the exponential phase. Therefore, 40 cycles were chosen for subsequent PCR amplifications of LIFR-β cDNA.

Conceptus Expression of LIFR-β mRNA

Photographs of agarose gels and autoradiograms of southern blots containing RT-PCR products from day 11 porcine conceptuses, for LIFR-β mRNA, are shown in Figure 4-2. Pictures of β_2m results are reproduced from Figure 3-2 for comparison. LIFR-β was amplified in 3 out of 6 litters, 1 spherical and 2 of filamentous morphology. There was no evidence of LIFR-β mRNA expression being morphology-dependent. LIFR-β was amplified from both spherical and filamentous day 11 conceptuses.

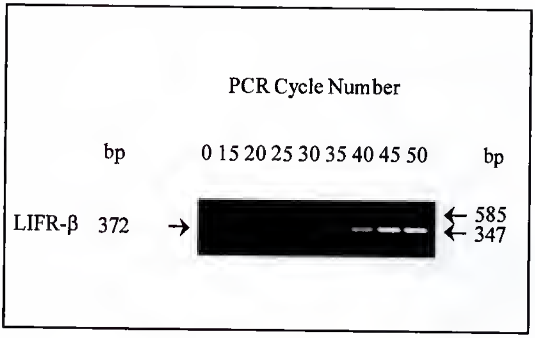


Figure 4-1. Ethidium bromide-stained agarose gel containing LIFR-β RT-PCR products from day 60 porcine placenta, amplified by 15 to 50 cycles of PCR.

The number of PCR cycles for obtaining LIFR product was 40. The numbers on the left are expected PCR product sizes. The numbers on the right are sizes of the DNA marker pUC18/Sau$_3$AI.

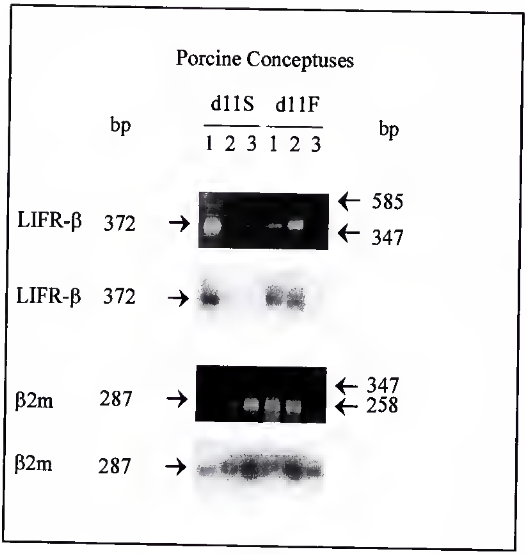


Figure 4-2. Ethidium bromide-stained agarose gels containing pools of day 11 conceptus LIFR-β and β$_2$m cDNAs, amplified by PCR. Southern blots of PCR products, hybridized with ^{32}P labeled probes for LIFR-β and β$_2$m, respectively.

The number of PCR cycles for obtaining LIFR product was 40. The numbers on the left are expected PCR product sizes. The numbers on the right are sizes of the DNA marker pUC18/Sau3AI. The results for β$_2$m are reproduced from Chapter-3 for comparison (d11S = day 11 spherical conceptuses; d11F = day 11 filamentous conceptuses; 1, 2 and 3 are different pools of conceptuses from each morphology group).

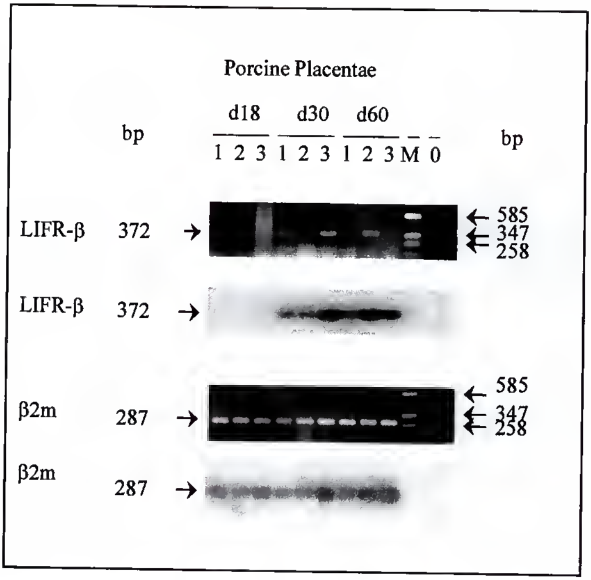


Figure 4-3. Ethidium bromide-stained agarose gels containing day 18, 30 and 60 placental LIFR-β and β_2m cDNAs, amplified by PCR. Southern blots of PCR products, hybridized with ^{32}P labeled probes for LIFR-β and β_2m, respectively.

The number of PCR cycles for obtaining LIFR product was 40. The numbers on the left are expected PCR product sizes. The numbers on the right are sizes of the DNA marker pUC18/Sau3AI. The results for β_2m are reproduced from Chapter-3 for comparison (d18 = day 18; d30 = day 30; d60 = day 60 placenta; 1, 2 and 3 represent different animals).

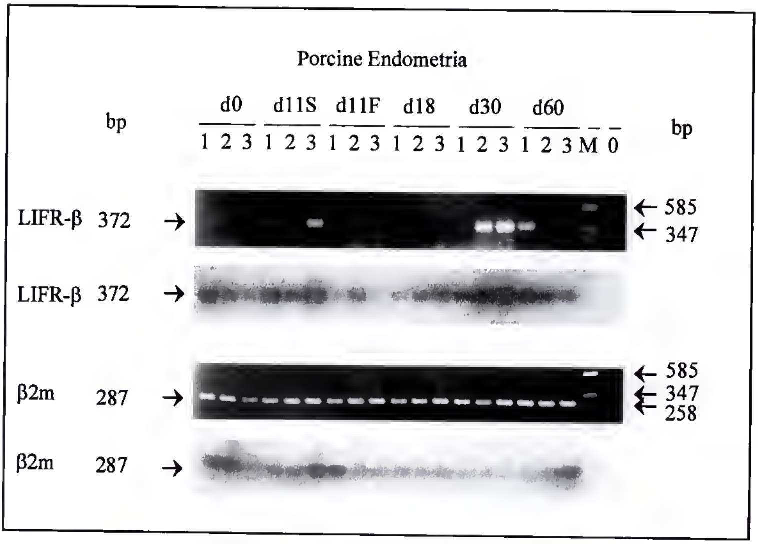


Figure 4-4. Ethidium bromide-stained agarose gels containing day 0, 11, 18, 30 and 60 endometrial LIFR-β and β_2m cDNAs, amplified by PCR. Southern blots of PCR products, hybridized with ^{32}P labeled probes for LIFR-β and β_2m, respectively.

The number of PCR cycles for obtaining LIFR product was 40. The numbers on the left are the predicted PCR product sizes. The numbers on the right are sizes of the DNA marker pUC18/Sau3AI. The results for β_2m are reproduced from Chapter-3 for comparison (d0 = day 0, d11S = day 11 endometria bearing spherical conceptuses; d11F = day 11 endometria bearing filamentous conceptuses d18 = day 18; d30 = day 30; d60 = day 60 endometria; 1, 2 and 3 represent different animals).

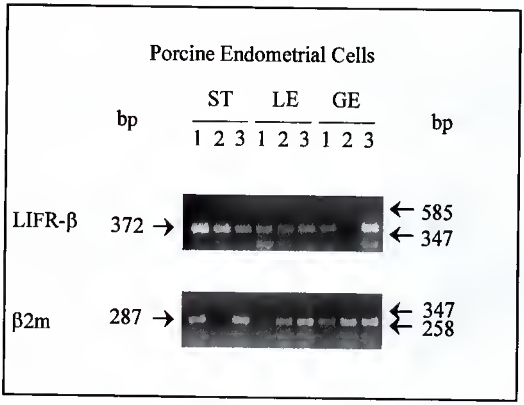


Figure 4-5. Ethidium bromide-stained agarose gels containing day 12 primary endometrial cell cultures' LIFR-β and β_2m cDNAs, amplified by PCR.
The number of PCR cycles for obtaining LIFR product was 40. The numbers on the left are expected PCR product sizes. The numbers on the right are sizes of the DNA marker pUC18/Sau3AI; 1, 2 and 3 are different animals. The results for β_2m are reproduced from Chapter-3 for comparison.

Table 4-2. Summary of results of RT-PCR for LIFR-β and β_2m mRNAs in porcine conceptuses, placentae, endometria, and endometrial cells.

Gene	**Conceptus**		**Placenta**			**Endometrium**						**Endometrial cells**		
	d11S	d11F	d18	d30	d60	d0	d11S	d11F	d18	d30	d60	ST	LE	GE
LIFR-β	+	+	-	++	++	+/-	++	+/-	+	+++	+	++	++	++

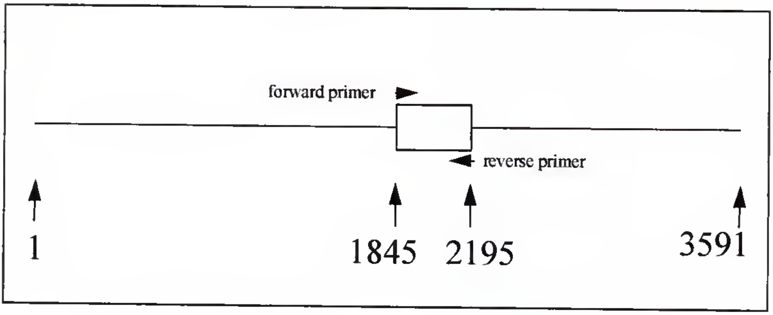


Figure 4-6. Schematic diagram of the cloned region of porcine LIFR-β cDNA.
The cDNA segment was cloned from day 60 porcine placenta. The total length of the porcine LIFR-β cDNA is unknown. Numbering is based on the human cDNA sequence (Gearing et al., 1991).

```
Ss    GGAAGCCTTT ACCCATTAAT GAAGCTTATG GAAAAATACT TTCTTATAAT
Hs    GGAAGCCTTT ACCCATTAAT GAAGCTAATG GAAAAATACT TTCCTACAAT
Mm    GGAAGCCTTT ACCTATTAAT GAAGCTAATG GAAAAATACT TTCCTACAAT

Ss    GTATCATATT CATCAGATGA GGAAACAAA. ..ACTTTCTG AGATTCCTGA
Hs    GTATCGTGTT CATCAGATGA GGAAACACAG TCCCTTTCTG AAATCCCTGA
Mm    GTTTCGTGTT CATTGAACGA GGAGACACAG TCAGTTTTGG AGATCTTCGA

Ss    TCCTCAACAC AGGGCAGAAT TACAACTTGA TAAAAATGAC TACATCATCA
Hs    TCCTCAGCAC AAAGCAGAGA TACGACTTGA TAAGAATGAC TACATCATCA
Mm    TCCTCAACAC AGAGCAGAGA TACAGCTTAG TAAAAATGAC TACATCATCA

Ss    GTGTGGTGGC AAAAAATTCT GCCGGCTCAT CACCACCTTC TAAAATAGCT
Hs    GCGTAGTGGC TAAAAATTCT GTGGGCTCAT CACCACCTTC CAAAATAGCG
Mm    GTGTGGTGGC AAGAAATTCT GCTGGCTCAT CACCACCTTC GAAAATAGCT

Ss    AGTATGGAAA TTCCCAATGA TGATCTCAAA ATAGAGCAGG CTCTTGGAAT
Hs    AGTATGGAAA TTCCAAATGA TGATCTCAAA ATAGAACAAG TTGTTGGGAT
Mm    AGTATGGAAA TCCCAAATGA TGACATCACA GTAGAGCAAG CGGTGGGGCT

Ss    GGGAAATAGG ATCCTCCTCA CTTGGAATTA CGACCCCAAC ATGACCTGCG
Hs    GGGAAAGGGG ATTCTCCTCA CCTGGCATTA CGACCCCAAC ATGACTTGCG
Mm    AGGAAACAGG ATCTTCCTCA CCTGGCGTCA CGACCCCAAC ATGACTTGTG

Ss    ACTATGTAAT TAAGTGGTGT AACTCATCTC AGTCTGGACC CTGCCTCATG
Hs    ACTACGTCAT TAAGTGGTGT AACTCGTCTC GGTCGGAACC ATGCCTTATG
Mm    ACTACGTAAT TAAATGGTGC AACTCATCTC GGTCTGAGCC CTGCCTCCTG

Ss    GACTGGAGAA AAGTTCCCTC AAACA
Hs    GACTGGAGAA AAGTTCCCTC AAACA
Mm    GACTGGAGAA AGGTTCCTTC AAACA
```

Figure 4-7. Nucleotide sequence from a cloned segment of porcine LIFR-β.
Porcine LIFR-β (Ss) sequences were aligned with human (Hs) and mouse (Mm) cDNAs. Shaded sequences are conserved between at least two species. PCR primer sequences are underlined.

```
Ss    KPLPINEAYG KILSYNVSYS SDEETK.LSE IPDPQHRAEL QLDKNDYIIS
Hs    KPLPINEANG KILSYNVSCS SDEETQSLSE IPDPQHKAEI RLDKNDYIIS
Mm    KPLPINEANG KILSYNVSCS LNEETQSVLE IFDPQHRAEI QLSKNDYIIS

Ss    VVAKNSAGSS PPSKIASMEI PNDDLKIEQA LGMGNRILLT WNYDPNMTCD
Hs    VVAKNSVGSS PPSKIASMEI PNDDLKIEQV VGMGKGILLT WHYDPNMTCD
Mm    VVARNSAGSS PPSKIASMEI PNDDITVEQA VGLGNRIFLT WRHDPNMTCD

Ss    YVIKWCNSSQ SGPCLMDWRK VPSN
Hs    YVIKWCNSSR SEPCLMDWRK VPSN
Mm    YVIKWCNSSR SEPCLLDWRK VPSN
```

Figure 4-8. Deduced amino acid sequence from a cloned segment of porcine LIFR-β. Porcine LIFR-β (Ss) sequences were aligned with human (Hs) and mouse (Mm) cDNAs. Shaded sequences are conserved between at least two species. Transcribed PCR primer sequences are underlined.

Placental Expression of LIFR-β mRNA

Figure 4-3 depicts photographs of agarose gels and autoradiograms of Southern blots for LIFR-β RT-PCR products obtained from total cellular RNA of days 18, 30 and 60 porcine placentae. Results for β_2m RT-PCR are reproduced from Figure 3-3, for comparison. The LIFR-β RT-PCR product was obtained from days 30 and 60 placentae.

Endometrial Expression of LIF, IL-6 and β_2m mRNAs

Figure 4-4 shows photographs of agarose gels and autoradiograms of Southern blots containing RT-PCR products from days 0, 11, 18, 30 and 60 porcine embryos for LIFR-β mRNA. Results for β_2m are reproduced from Figure 3-4 for comparison. LIFR-β RT-PCR product was observed in day 0, 11 and 60 endometria, but much more intensively in day 30 endometria.

Endometrial Cell Expression of LIFR-β mRNA

Figure 4-5 shows photographs of agarose gels containing RT-PCR products from day 12 porcine endometrial cells (ST, LE and GE), amplified using primers for LIFR-β mRNA. LIFR-β RT-PCR products were observed from most of the cells examined. Moreover, there was no evidence of differential LIFR-β expression with regard to the cell type.

Cloning and Sequencing

The cloned LIFR-β PCR product was 272 bp long, although its equivalent in mouse and human was 275 nucleotides long, due to a three base gap in the porcine sequence, as compared to human and mouse sequences. A diagram of the cloned region of porcine LIFR-β cDNA is shown in Figure 4-6. Aligned porcine, human and mouse nucleotide and amino acid sequences of LIFR-β cDNA are shown in Figure 4-7. Percentages of similarity of nucleotide and deduced peptide sequences between human, mouse and swine LIFR-β, are shown in Table 4-3. The cloned segment of LIFR-β cDNA is 87% similar to the corresponding segment of LIFR-β cDNA from the human (Gearing et al., 1991) and 83% from the mouse (Tomida et al., 1993). The deduced amino acid sequence is 93% similar to human and 91% to mouse LIFR-β.

Discussion

RT-PCR is a method limited by the fact that the amplification of DNA is exponential, therefore a little variations in initial amount of mRNA between samples can result in great differences in the final product (Dukas et al., 1993). To prevent large variations of product resulting from the same amount of mRNA, positive and negative controls were used. Negative PCR control tube contained all components as the sample mixtures except template cDNA. Primers for β_2m were used to amplify positive controls. Sometimes, variations in the amount of PCR products between animals were observed. It is not known for each particular sample whether the source of the variation was the method or biological variations between the animals, such as developmental stage, number of conceptuses, endocrine profiles or feto-placental weights. Another limitation of RT-PCR is the fact that this method detects mRNA, and not protein, the final product of gene expression. In some instances, a major regulation of gene expression takes place after mRNA has been synthesized (Raqib, 1996).

The LIFR-β mRNA was detected in 3 of 6 pools of day 11 conceptuses, regardless of their morphology. Such sporadic expression may be due to the possibility that LIFR-β gene expression is borderline for the sensitivity of detection by the methods used in this study. It is not known what regulates LIFR-β expression in porcine conceptuses, or in other tissues, since regulation of LIFR-β expression has not been extensively studied. Since LIF is expressed neither in porcine day 11 conceptuses (Chapter 2), nor in day 14 trophoblast (Chapter 6), LIFR-β expression in conceptuses may be regulated by molecules present in the surrounding ULF. Some candidate LIFR-β regulators are steroid hormones (Bazer et al., 1991) and growth factors (Simmen et al., 1995). In other systems, cytokine receptors have been reported to be modulated by their, or other, ligands. For example, IL-6R-α transcription is inhibited by LIF in myelomonocytic leukemic M1 cells (Yamaguchi et al., 1992), while IL-6 downregulates gp130 in myeloma and melanoma cells (Schwabe et al., 1994). On the other hand, recombinant mouse (rm)LIF (100-10,000 U/ml), as well as rh IL-6 (100-10,000 U/ml) did not result in altered IL-6R-α and gp130 receptor expression in a megakaryoblastic cell line (Sasaki et al., 1995). IL-6 induces the expression of mRNA for gp130 in HepG2 cells (Schooltink et al., 1992). Since LIFR-β is structurally and evolutionary closer to gp130 than to IL-6R-α (Gearing et al., 1991), it is possible that IL-6 or LIF modulate LIFR-β expression. It is also possible that the ligand induces the expression of its own receptor, as is the case for upregulation of IL-6R-α by IL-6 in rat liver (Geisterfer et al., 1995), and in two human multiple myeloma cell lines (Lasfar et al., 1994).

The function of LIFR-β at this developmental time can only be speculated on. These function(s) could be the same as in the mouse and human blastocysts, where LIFR-β mediates the induction of trophoblast proliferation by LIF (Lavranos et al., 1995). LIFR-β could also mediate differentiation of porcine inner cell mass, the same effect being observed for mouse embryos (Pease et al., 1990). The suppression of porcine embryonic stem cell differentiation by LIF, observed by Hochereau de Reviers and Perreau (1993), is likely to be mediated by LIFR-β. However, the method used in the present study can not differentiate between LIFR-β expression in trophoblast versus inner cell mass, therefore it is not possible to assign the cell type(s) in which LIFR-β mRNA is expressed.

LIFR-β mRNA was detected in all days 30 and 60, but not in day 18 placentae. It was demonstrated in Chapter-3 that mRNA expression of IL-6 gene was detectable in day 18 placenta, with IL-6 mRNA being most abundant in day 30 and 60 placenta. It is unknown whether IL-6 may downregulate LIFR-β, but if true, this mechanism could be responsible for the fact that in day 18 placenta, LIF mRNA is detectable (although barely), whereas LIFR-β mRNA is not. It is also possible that LIF downregulates the expression of its own receptor, like IL-6 does with IL-6Rα in multiple myeloma cells (Lasfar et al., 1994). There is a possibility that other cytokines of the hemopoietic group are expressed in porcine placenta or endometrium, and which induce LIFR-β. This would probably be true for OSM (Davis et al., 1993) and CNTF (Rose et al., 1994), since they both utilize LIFR-β. On the other hand, there are other cytokines, the expression of which is not coincident with the expression of their receptors. For example, G-CSF is another cytokine from the hematopoietic group, believed to be involved in paracrine regulation of trophoblast growth or function during placentation. G-CSF, is synthesized by human trophoblast throughout pregnancy, but its receptor is expressed in placenta only during the first and third trimester (McCracken et al., 1996).

The LIFR-β mRNA was expressed in porcine endometrium on all days examined. However, it was very low in day 11 pigs bearing filamentous conceptuses and in days 0 and 18 endometrium. Signal was the highest in day 30 endometrium, followed by day 60 endometrium and day 11 endometrium exposed to spherical conceptuses only. It appears that physiologically significant amounts of LIFR-β mRNA are found only in endometrium following implantation. At this time of pregnancy, antileukoproteinase expression is also high (Simmen et al., 1992), which suggests that LIF might induce the expression of this gene. Uteroferrin expression is also high at this time (Simmen et al., 1988). There is a high availability of ligand at this time, in both endometrium and placenta. This may suggest that signaling is taking place within endometrium. Since LIF mRNA is most abundant in day 30 placenta, and if LIF can cross the feto-maternal barrier, the LIFR-β mediated signaling may take place in the direction from fetal to maternal tissues. It is not known whether LIF crosses the feto-maternal barrier in swine, but a number of other peptides are known to do so. These include uteroferrin (Roberts et al., 1986), antileukoproteinase (Simmen et al., 1992) and retinol binding protein (Harney et al., 1994).

LIFR-β mRNA was detected in all three endometrial cell types ST, LE and GE, with no differences in relative steady state levels found. These findings differ from data for the humans, where LIFR-β is expressed during proliferative and secretory phases of the cycle, and is restricted to the LE (Cullinan et al., 1996). It is possible that different species have different LIFR-β expression patterns. Since primary endometrial cell cultures are not

100% pure, it is also possible that other cell types were contaminated by LE. If contamination was not a source of LIFR-β PCR product in GE and ST, these findings may suggest that all three endometrial cell types are responsive to the LIF signal, from either placenta or endometrium. These *in vitro* experiments did not completely mimic the *in vivo* conditions, especially with respect to interactions with other two cell types and the conceptus. Each endometrial cell type was cultured separately from other cell types, which may contribute to differential expression. Likely, a slight difference in intensity between the LIFR-β signal from fresh endometria exposed to spherical vs. filamentous conceptuses, may have been induced by a factor secreted by the conceptus, which was absent in the culture. The second band of the smaller size then expected for LIFR-β may be an artefact resulting from non-specific primer binding, or a result of self-priming. The PCR primers have homology to the region on LIFR-β gene that encodes extracellular receptor domain, and thus detects both mRNAs for the soluble and membrane-bound receptor. Therefore, an additional band is probably not a result of differential splicing.

The cloned segment of the porcine LIFR-β encodes a part of the extracellular domain. The degree of conservation of this segment between porcine, human and mouse sequence is relatively high. Porcine sequence is 83% similar to the corresponding segment of the mouse and 87% of the human sequence. This may partly be a result of the higher degree of conservation of the cloned segment compared to the whole cDNA, since the regions of high conservation between human and mouse LIFR-β cDNAs were used for designing the PCR primers. The comparison study between LIF cDNA sequences from the same three species revealed similar degree of conservation between different species. The coding region of the porcine LIF is 78% identical to mouse and 87% identical to human sequences of the coding regions on the LIF cDNAs (Gough et al., 1992). There was one codon gap of three bases in the porcine LIFR-β cDNA sequence, compared to human and mouse gene, namely bases 81 to 82 of the cloned fragment, or bases 1747 to 1749 of the coding region, were missing in swine, resulting in the deletion of the amino acid serine at the position 583, which is encoded by the human (Gearing et al., 1991) and mouse (Tomida et al., 1993) cDNA. Since this gap is for the three nucleotide bases, it did not cause frame shifting. It is not clear whether the gap really existed in the gene, or it was PCR-generated. Since LIFR-β, and many other genes, are more conserved between pig and human, than between any of these species versus mouse, it is more likely that the gap was PCR generated. On the other hand, the gap occurred within the codon, not affecting neighboring codons, which increases the likelihood that the gap is not a PCR artefact.

In pig, LIF may have a greater role after blastocyst implantation, although it is required for the implantation the mouse (Stewart et al., 1992), and possibly human (Cullinan et al., 1996) blastocysts. LIFR-β may mediate inhibition of ICM differentiation and / or trophoblast proliferation in pig. The fact that LIFR-β is highly expressed in later stages of pregnancy, by endometrium and that the LIF mRNA expression is the highest in day 30 placenta, indicates its possible role in feto-maternal communication at the later stages of pregnancy. The 372 bp LIFR-β cDNA segment has been cloned, which can be used as a probe for subsequent experiments. Its sequence is conserved between porcine, human and mouse cDNAs.

CHAPTER 5
IN VITRO EFFECTS OF LEUKEMIA INHIBITORY FACTOR AND INTERLEUKIN-6 ON THE EXPRESSION LEVELS OF ANTILEUKOPROTEINASE mRNA IN PORCINE ENDOMETRIUM

Introduction

LIF and IL-6 are members of the hematopoietic group of cytokines. LIF (Bhatt et al., 1991; Yang et al., 1994; Anegon et al., 1994; Brisson et al., 1996) and IL-6 (Tabibzadeh et al., 1989; Motro et al., 1990; Anegon et al., 1994) are expressed in the endometrium of various species, including swine. In addition, LIF (Conquet and Brulet, 1990; Murray et al., 1990) and IL-6 (Murray et al., 1990; De et al., 1992) transcripts are expressed in preimplantation mouse blastocysts and early postimplantation placentas. IL-6 mRNA has also been identified in human (Stephanou et al., 1995), sheep, cow and swine (Mathialagan et al. 1992) periimplantation embryos. The presence of IL-6 has been reported in days 4 and 11 porcine ULF in concentrations of 6 and 14 thousands U/ml, respectively (Anegon et al., 1994). This factor stimulates cytochrome P450 aromatase gene expression in adipose tissue (Zhao et al., 1995) and in human granulosa cells, in the presence of macrophages (Machelon et al., 1994), although recombinant human (rh)IL-6 (200 U/ml) had no effect on progesterone and estrogen synthesis in porcine large and small luteal cells (Pitzel et al., 1993). Recombinant human (rh) IL-6 (100 U/ml) induced the expression of alpha$_2$macroglobulin (α_2m) in rat hepatocyte primary cultures (Andus et al., 1988). Alpha$_2$m is a broad spectrum protease inhibitor associated with inflammatory responses and is associated with remodeling phenomena in the rat ovary (Gaddy-Kurten et al., 1989). IL-6 has been found to upregulate proteins not previously identified as APRF, such as alpha-fetoprotein, β_2m, and fibronectin in fetal rat hepatocytes (Roncero et al., 1995). RhIL-6 (100 - 1000 ng/ml) stimulates collagen glycosaminoglycan production by cultured human dermal fibroblasts (Duncan and Berman, 1991). All these changes have a documented or presumed role in pregnancy. Anegon et al. (1994) reported levels of 13,000 U/ml LIF in day 12 pregnant porcine ULF. It is possible that LIF has similar effects as IL-6, since the biological endpoints of these two cytokines often overlap, presumably due to utilization of a common receptor component, gp 130 (Piquet-Pelorce et al., 1994).

Human elastase is a 218 amino acid proteinase (Sinha et al., 1987). Elastase (Stack et al., 1992; Mayer et al., 1993; Bussolino et al., 1994) and other proteinases, including metalloproteinases (Behrendtsen et al., 1992), gelatinases (Talhouk et al., 1991), plasminogen (Stack et al., 1992) and trypsin (Bicsak et al., 1989) have been associated with extracellular-matrix remodeling during various physiological and pathological processes. The expression of proteinases, like collagenase (Woessner, 1979), cathepsin-D (Barth and Afting, 1984), arginine esteropeptidase (Jazin et al., 1988), MMP-9, and urokinase-type plasminogen activator uPA (Harvey et al., 1995), in the uterus has been reported and may be important in pregnancy.

ALP also called SLPI, or mucus proteinase inhibitor (MPI), is a lysosomal inhibitor of the serine proteinases, elastase and cathepsin-G. The human ALP gene was cloned by Thompson and Ohlsson (1986). The carboxyl terminal position of ALP is associated with its proteinase activity (Thompson and Ohlsson, 1986), while the amino terminal domain is involved in interactions with proteinases (Ying et al., 1994). ALP is abundantly expressed by bronchial epithelial cells (Sallenave et al., 1994). Lungs are the tissue site where neutrophils secrete large amounts of elastase, and more so during inflammation (Maruyama et al., 1994). Furthermore, the number of ALP producing cells is positively correlated with bronchiole inflammation and the level of airway epithelium destruction (Kramps et al., 1988). Therefore, ALP may protect the airway epithelium from local degradation by neutrophil elastase.

There are indications that ALP plays an important role in pregnancy. ALP is abundantly expressed in uteri of species exhibiting epitheliochorial placentation, in contrast to those species exhibiting hemo- or endotheliochorial placentation type. Because of this, and the fact that porcine conceptuses are invasive at ectopic sites (Samuel et al., 1971), it has been postulated that ALP expression is a major maternal mechanism of resisting trophoblast invasiveness (Badinga et al., 1994). It is not known what regulates the expression of ALP in the pregnant uterus. In uteri of prepubertal gilts, ALP gene expression is positively modulated by progesterone and estrogen (Farmer et al., 1990), but the main regulator during pregnancy has not been identified. One possibility is that ALP gene expression in porcine endometrium is regulated by cytokines, as is the case for human airway epithelial cells, where ALP expression is positively regulated by IL-1 and TNF-α (Sallenave et al., 1994). These two cytokines also induced the expression of LIF (Lubbert et al., 1991) and IL-6 (Norris et al., 1994; Rifas et al., 1995), which may suggest that the effect of IL-1 and TNF-α on ALP expression is indirect, via induction of IL-6 as an intermediary stimulator. It is not known however, whether IL-6 or TNF-α induces the synthesis of ALP in porcine uterus. In mouse endometrium, IL-1 mRNA is abundant at proestrus and estrus (De et al., 1992), on day 1 of pregnancy (Kover et al., 1995), and together with TNF-α, during periimplantation period (De et al., 1993).

LIF and IL-6 expression in pig endometrium (Chapter 3) is correlated with the temporal pattern of ALP expression (Farmer et al., 1990). LIF mRNA expression is high in day 30 endometrium and day 30 and 60 placenta, while IL-6 mRNA is abundantly expressed in day 30 and 60 endometrium. The expression of both mRNAs is very low or undetectable in day 18 endometrium. Antileukoproteinase (ALP) gene expression is low in early porcine pregnancy, and high in mid- and late pregnancy (Farmer et al., 1990). Because of ALP being coexpressed with LIF and IL-6 (Chapter 3) and LIFR-β (Chapter 4) in day 30 porcine endometrium and placenta, and the indication that LIF and IL-6 may be intermediary modulators of ALP expression, it is postulated that these two hematopoietic cytokines have a regulatory role in endometrial ALP gene expression. Thus, LIF and IL-6 may help determine the type of placentation that occurs.

The use of endometrial explant cultures is a powerful tool in studying biological effects of many substances on endometrium (Dudley et al., 1992). Endometrial explants can be used to test the effects of cytokines on the expression of genes normally expressed in uterus. In order to test the above hypothesis, day 12 and 21 endometrial explants were used, which are not expected to express LIF or IL-6. After treating explants with the two cytokines, we measured steady state levels of ALP mRNA.

Materials and Methods

Animals

Prepubertal crossbred gilts were obtained and subsequently mated on their second or later estrus after cyclicality commenced. The day of onset of estrus was considered as day 0. Three animals were slaughtered on day 12 and three on day 21 of pregnancy at the Meats Processing Facility (University of Florida). After exsanguination, reproductive tracts were dissected and immersed in ice. Uteri were transferred to a laminar flow hood where the uterine horns were trimmed from the mesometrium and blotted free of blood. Uteri were flushed with sterile PBS, pH 7.2.

Explant culture

The porcine endometrial explant culture method employed by Murray et al. (1989) was used. Several sections were taken from various parts of uterine horns of a freshly killed gilt. Endometrium was separated in the form of tiny strips and pooled together in PBS containing 1% ABAM (Sigma, St. Louis, MO). Then, 250 mg of tissue was weighed out and put into each culture well in a (Multiwell) 12-well culture plate with low evaporation lid (Becton Dickinson Labware, Lincoln Park, NJ) filled with 2 ml of minimal essential medium (MEM) with Earl's salts (GIBCO BRL; Gaithersburg, MD), modified by the addition of 1% ABAM (GIBCO BRL; Gaithersburg, MD), 1% MEM vitamin solution (GIBCO BRL; Gaithersburg, MD), 1.5 g glucose / 500 ml and 0.146 g / L-glutamine 500 ml, and filter sterilized through 0.2 μm into an autoclaved bottle (pig MEM). Two to three wells were used per treatment, depending on the number of replicates per experiment. Explants were incubated for 1.5 hr in pig MEM, at 37°C in an atmosphere of 95% air and

5% CO_2. After initial incubation of 1.5 hr, the MEM was replaced with treatment medium (2 ml pig MEM with the addition of cytokines). Explants from each pig received a set of 10 treatments in 2 to 3 replicates per pig. The same amounts of fresh endometria were also taken in 2 to 3 replicates per pig. Explants from each pig were treated with hrLIF (Sigma, St. Louis, MO) at concentrations of 10 and 100 ng/ml (about 86 and 860 U/ml) and hrIL-6 (Sigma, St. Louis, MO) at concentrations of 10 and 100 ng/ml (about 19 and 190 U/ml). Duration of treatment was 6 or 24 hours. Untreated explants were also treated for 6 or 24 hours in 2 to 3 replicates per animal.

Northern and Dot Blot Analysis

Total cellular RNA was isolated by adding 3 ml of TRIzol reagent (GIBCO BRL; Gaithersburg, MD) to each endometrial explant, and homogenizing briefly, following the protocol provided with the kit, except that RNA was precipitated with isopropanol at -80°C overnight. Twenty µg of total RNA per lane was electrophoresed in a 1.5% agarose-formaldehyde gel and transferred to a Biotrans nylon membrane (ICN Biochemicals; Aurora, OH), using the TurboBlotter system (Schleicher and Schuell, Keene, NH). Ten µg total RNA was transferred by Minifold dot blot apparatus (Schleicher & Schuel, Keene, NH) onto a Biotrans nylon membrane and cross linked to the membrane by exposure to UV light for 2 minutes.

Porcine ALP (bp 9-408) cDNA and porcine β_2m (PCR cloning described in Chapter 3) DNA were gel-purified and nick-translated as described below. About 200 ng gel-purified ALP and β_2m cDNA fragments were radiolabeled using a Nick Translation kit by Amersham Life Sciences (Arlington Heights, IL) and 50 µCi [^{32}P] α-dCTP (3,000

Ci/mmol) and the probes were purified from unincorporated radioisotope by gel filtration chromatography on Sephadex G-50 columns (Pharmacia, Piscataway, NJ). The Northern membrane was hybridized at 42°C in 50% formamide, 4x SSC, 1x Denhardt's solution, 0.1% SDS, yeast RNA (250 μg/ml), pH 6.5, overnight. The membrane was washed twice at 50°C in 2X SSC / 0.1% SDS for 20 minutes, and once at 55°C in 0.1X SSC / 0.1% SDS for 20 minutes, and exposed to ISO 9000 X-ray film (DuPont NEN, Boston, MA) for 48 hours at -80°C. The migration distance of the ALP-hybridized RNAs was compared to the migration distances of known RNAs: 28S and 18S ribosomal RNAs. The dot-blot membranes were hybridized with ALP cDNA in the same manner and exposed to ISO 9000 X-ray film (DuPont NEN, Boston, MA) for 2-3 days. Quantification of labeling intensity was performed using a PhosphorImager Series 400 System (Molecular Dynamics, Sunnyvale, CA). A Molecular Dynamics Software package (Image Quant V3.3) was used to process the data. The background values were subtracted from each sample. After quantification, membranes were stripped in 1l of 1mM TRIS, 1 mM EDTA and 0.1X Denhardt solution twice for 1 hour at 75°C, then washed in 0.1X SSC and hybridized with the β_2m cDNA probe.

Statistical Analysis

The radioactivity volume from each analysis of RNAs were analyzed by method of least-squares analysis of variance using the General Linear Models procedures of PC-SAS (SAS, 1988). Mathematical model included treatment, dose, culture time, and interactions. There were 7 samples per treatment for day 12 (n = 77), and 8 observations per treatment for day 21 endometrium (n = 88; the total of 165). For each sample, there was one observation for each parameter: ALP and β_2m probes radioactivity intensities (the total of 330 observations).

Results

Northern Blot Analysis

Northern blot analysis of endometrial explants was performed in order to demonstrate that the hybridization signal on the dot-blot membranes represents ALP mRNA. Figure 5-1, top panel, shows an ethidium bromide stained, formaldehyde agarose gel in which was electrophoresed representative endometrial total RNAs. The bottom panel of the same figure shows the resultant Northern blot of the same gel. The size of the hybridized RNAs was estimated to be about 800 nucleotides, which is the size of the ALP mRNA.

RNA Dot-Blot Analysis

In Figure 5-2 (top panel) is shown a typical dot-blot of pregnant porcine day 21 endometrial RNAs from the treatment groups, hybridized with the radiolabeled ALP probe. Total cellular RNA from each animal was dot-blotted on a separate membrane, resulting in 3 blots per day of pregnancy, an the total of 6 membranes. The bottom panel shows the same dot-blot, stripped and rehybridized with the radiolabeled β_2m probe.

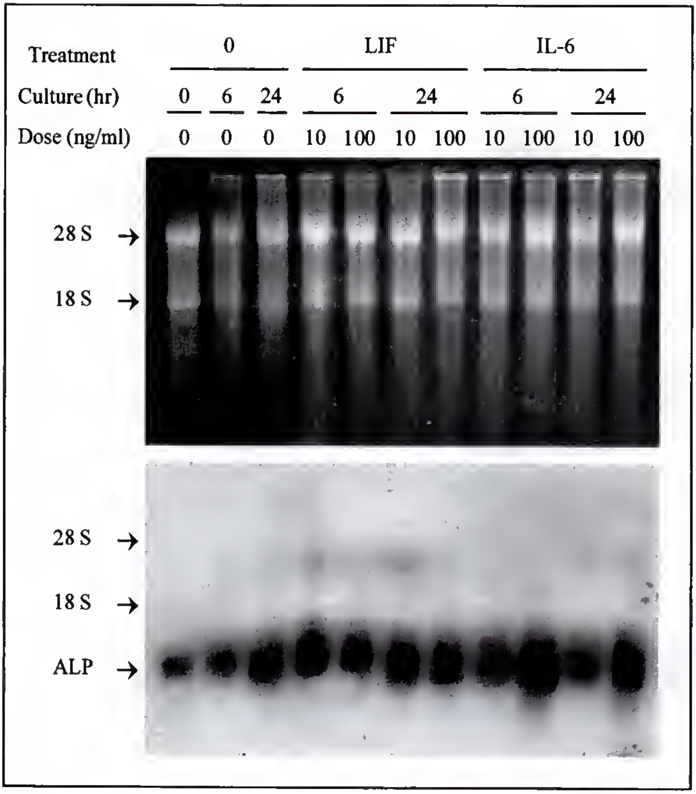


Figure 5-1. Formaldehyde agarose gel containing electrophoresed pregnant porcine day 21 endometrium (20 μg/lane), from explants treated with LIF and IL-6 (top panel). Autoradiograph of Northern blot of the same gel, hybridized with radiolabeled ALP probe (bottom panel).

The size of the hybridized RNA is about 800 nucleotides, which is the size of ALP mRNA.

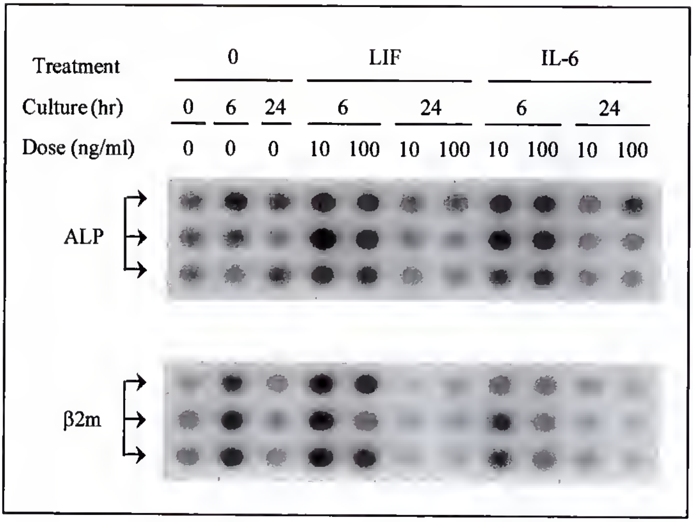


Figure 5-2. A representative RNA dot-blot of pregnant porcine day 21 endometrium (20 μg/dot) from one animal, treated with LIF and IL-6 and hybridized with radiolabeled ALP probe (top panel). The same dot-blot, stripped and rehybridized with radiolabeled β_2m probe (bottom panel).

The three samples are replicates of the experiment from the same animal.

Table 5-1. ANOVA table of results from the cytokine-untreated endometria. Dependent variable = ALP radioactivity volumes.

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F
MODEL	9	71703.266179	7967.029575	13.46	0.0001
ERROR	35	20715.844719	591.881278		
CORRECTED TOTAL	44	92419.110898			

R-SQUARE	0.775849
C.V.	44.52509
ALP MEAN	54.640222

SOURCE	DF	TYPE III SS	MEAN SQUARE	F VALUE	PR > F
DAY	1	54145.574779	54145.574779	91.48	0.0001
PIG (DAY)	4	5161.285555	1290.321389	2.18	0.0915
HR.	2	2234.997750	1117.498875	1.89	0.1240
DAY (HR.)	2	3634.866452	1817.433226	3.07	0.0591

Table 5-2. ANOVA table of results from the cytokine-untreated endometria. Dependent variable = β_2m radioactivity volumes.

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F
MODEL	9	59037.515701	6559.723967	28.94	0.0001
ERROR	35	7932.707735	226.648792		
CORRECTED TOTAL	44	66970.223436			
R-SQUARE	0.881549				
C.V.	26.32020				
β_2m MEAN	57.198889				
SOURCE	DF	TYPE III SS	MEAN SQUARE	F VALUE	PR > F
DAY	1	9428.459520	9428.459520	41.60	0.0001
PIG (DAY)	4	47453.506041	11863.376510	52.34	0.0001
HR.	2	1419.503313	709.751656	3.13	0.0561
DAY (HR.)	2	1745.978989	872.989495	3.85	0.0308

Table 5-3. Summary of ALP and β_2m results in explants not treated with cytokines, cultured for 0, 6 and 24 hours.

Probe	**Day**	**0 hr**	**6 hr**	**24 hr**
ALP	**12**	12.91 (1.95)	18.11 (1.95)	20.46 (1.95)
ALP	**21**	96.01 (11.63)	102.11 (11.63)	65.79 (11.63)
β_2m	**12**	31.64 (4.61)	35.13 (4.61)	39.25 (4.61)
β_2m	**21**	59.09 (6.10)	80.91 (6.10)	54.66 (6.10)

Day	**Orthogonal Contrast**	**ALP**	**β_2m**
12	**0 hr vs. 6 and 24 hr**	< 0.02	> 0.34
12	**6 hr vs. 24 hr**	> 0.39	> 0.53
21	**0 hr vs. 6 and 24 hr**	> 0.40	> 0.25
21	**6 hr vs. 24 hr**	< 0.04	< 0.02

Hybridization intensities (in arbitrary units) of dot-blots with total cellular RNA from cytokine untreated day 12 and 21 pregnant porcine endometria cultured for 0, 6 and 24 hours, and hybridized with radioactive ALP, then stripped and re-probed with β_2m probes. Results of experiments with 3 animals for each day of pregnancy, repeated 2 or 3 times for each animal. LS means (± SEM), top panel. Orthogonal contrasts, bottom panel.

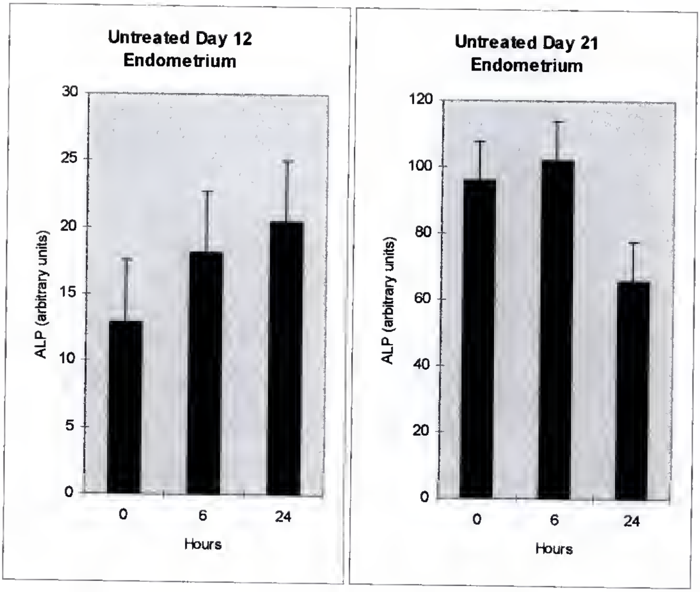


Figure 5-3. The effects of duration of endometrial explant culture on ALP gene expression in day 12 and day 21 pregnant, cytokine-untreated porcine endometrium.

X axis represents duration of culture; Y values are LS means of radioactivity volumes. Bars represent LS SEM. Each graph is a result of experiments with 3 animals, repeated 2 or 3 times each. Orthogonal contrast analysis demonstrated significant differences between cultured and uncultured endometria within day 12 pregnant endometria, at the $p<0.02$ level, but not within day 21 pregnant endometria ($p>0.05$).

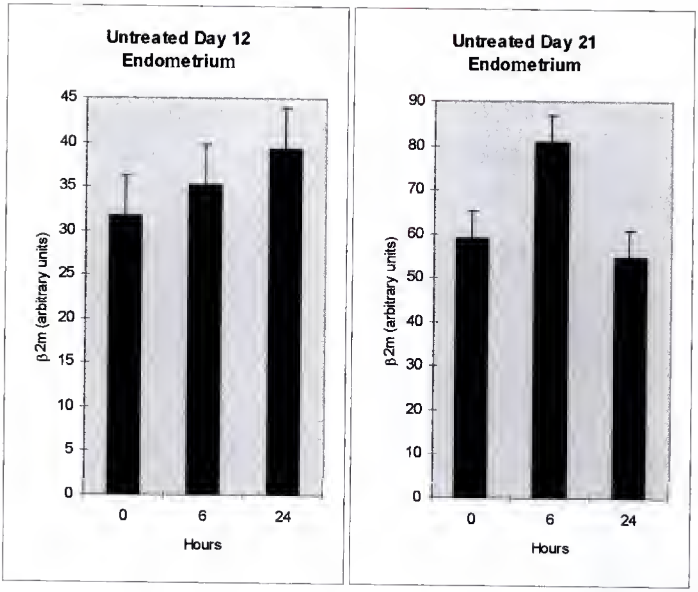


Figure 5-4. The effect of duration of culture on β_2m gene expression in day 12 and day 21 pregnant, cytokine-untreated porcine endometrium.

X axis represents duration of culture; Y-values are LS means of radioactivity volumes. Bars represent LS SEM. Each graph is a result of experiments with 3 animals, repeated 2 or 3 times each. The mRNA expression was 80% higher in day 21 explants, compared to day 12 explants ($p<0.01$). Orthogonal contrast analysis did not show significant differences between fresh endometrium and cultured explants within day 12, nor day 21 pregnant endometria, at the $p\leq 0.05$ level.

Table 5-4. ANOVA table of results from the cytokine-treated endometria. Dependent variable = ALP radioactivity volumes.

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F
MODEL	59	340423.23079	5769.88527	10.92	0.0001
ERROR	90	47561.11052	528.45678		
CORRECTED TOTAL	149	387984.34131			
R-SQUARE	0.877415				
C.V.	38.73569				
ALP MEAN	59.346267				
SOURCE	DF	TYPE III SS	MEAN SQUARE	F VALUE	PR > F
DAY	1	173919.85937	173919.85937	329.11	0.0001
PIG (DAY)	4	12209.11886	3052.27972	5.78	0.0003
TREAT	9	32404.14493	3600.46055	6.81	0.0001
DAY (TREAT)	9	46211.68287	5134.63143	9.72	0.0001
PIG (DAY*TREAT)	36	45042.52356	1251.18121	2.37	0.0005

Table 5-5. ANOVA table of results from the cytokine-treated endometria. Dependent variable = β_2m radioactivity volumes.

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F VALUE	PR > F
MODEL	59	228984.25667	3881.08910	19.92	0.0001
ERROR	90	17532.84554	194.80939		
CORRECTED TOTAL	149	246517.10221			
R-SQUARE	0.928878				
C.V.	24.35086				
β_2m MEAN	57.317953				
SOURCE	DF	TYPE III SS	MEAN SQUARE	F VALUE	PR > F
DAY	1	46811.64960	46811.64960	240.29	0.0001
PIG (DAY)	4	113946.86590	28486.71647	146.23	0.0001
TREAT	9	15993.69651	1777.07739	9.12	0.0001
DAY (TREAT)	9	14053.30395	1561.47822	8.02	0.0001
PIG (DAY*TREAT)	36	26754.98040	743.19390	3.81	0.0001

Table 5-6. Summary of results, explants treated with 0, 10 and 100 ng/ml of cytokines.

Day	**Dose**	**LIF**		**IL-6**	
	ng/ml	**ALP**	**β_2m**	**ALP**	**β_2m**
12	**0**	19.45 (2.39)	37.06 (7.38)	19.45 (2.39)	37.06 (7.38)
12	**10**	19.38 (2.39)	39.17 (7.38)	26.12 (2.39)	25.57 (7.38)
12	**100**	26.62 (2.39)	38.36 (7.38)	28.04 (2.39)	24.90 (7.38)
21	**0**	84.28 (10.09)	68.01 (11.44)	84.28 (10.09)	68.01 (11.44)
21	**10**	102.91 (10.09)	95.28 (11.44)	82.58 (10.09)	80.55 (11.44)
21	**100**	92.10 (10.09)	51.85 (11.44)	105.28 (10.09)	49.68 (11.44)

Day	**Orthogonal Contrast**	**LIF**		**IL-6**	
		ALP	**β_2m**	**ALP**	**β_2m**
12	**10 and 100 ng/ml VS. 0 ng/ml**	< 0.01	> 0.71	< 0.01	< 0.02
12	**10 VS. 100 ng/ml**	< 0.01	> 0.73	> 0.18	> 0.77
21	**10 and 100 ng/ml VS. 0 ng/ml**	> 0.17	> 0.22	> 0.31	> 0.52
21	**10 VS. 100 ng/ml**	> 0.33	> 0.23	< 0.05	< 0.06

Hybridization intensities (in arbitrary units) of dot-blots with total cellular RNA from day 12 and 21 pregnant porcine endometria treated with 0, 10 and 100 ng/ml LIF or IL-6. Dot-blots were hybridized with radioactive ALP, stripped and re-probed with β_2m probes. Results of experiments with 3 animals for each day of pregnancy, repeated 2 or 3 times for each animal. LS means (± SEM), top panel. Orthogonal contrasts, bottom panel.

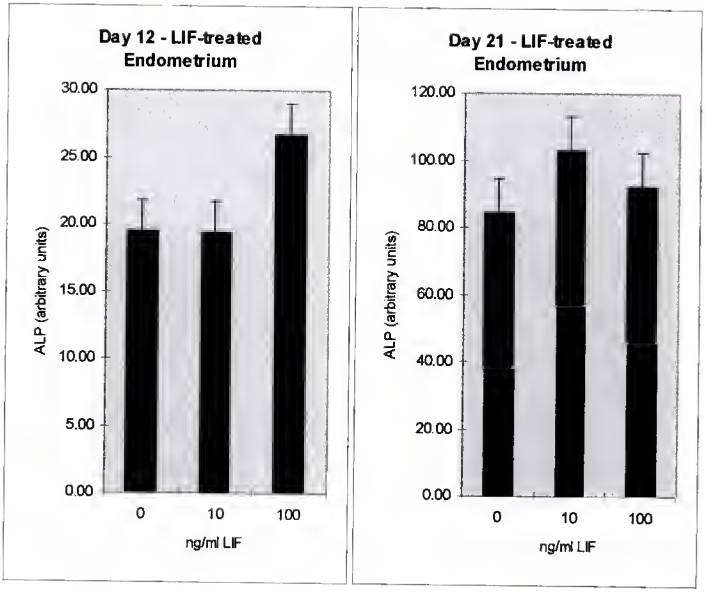
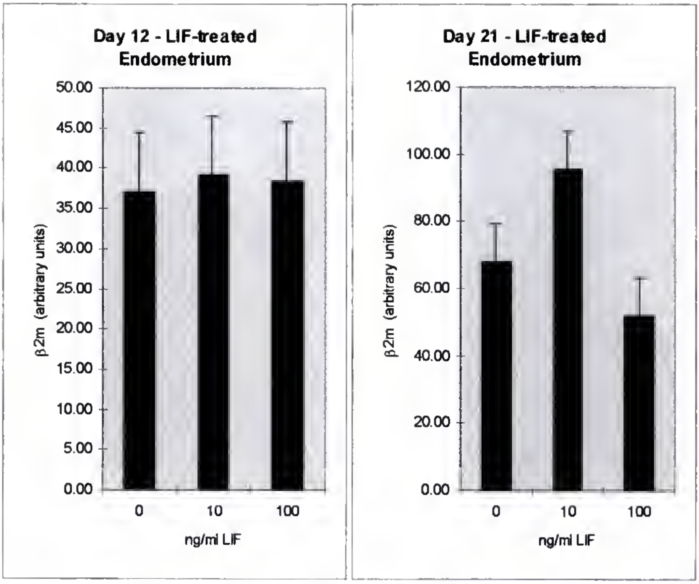


Figure 5-5. The effect of LIF on the level of ALP mRNA in days 12 and 21 porcine endometrial explants.

X-values are days of pregnancy; Y-values are LS means of radioactivity volumes. Error bars represent LS SEM. Each graph is a result of experiments with 3 animals, repeated 2 or 3 times each. By orthogonal contrast analysis, ALP radioactivity volume was significantly different ($p<0.01$) in 100 ng/ml LIF-treated day 12 explants, versus untreated explants and those treated with 10 ng/ml LIF. In day 21 explants, no significant differences were detected at the 0.05 level.



Day 12 - LIF-treated Endometrium

Day 21 - LIF-treated Endometrium

Figure 5-6. The effect of LIF on the level of β_2m mRNA in days 12 and 21 porcine endometrial explants.

X-values are days of pregnancy; Y-values are LS means of radioactivity volumes. Error bars represent LS SEM. Each graph is a result of experiments with 3 animals, repeated 2 or 3 times each. By orthogonal contrast analysis, β_2m signal was not significantly different between the two doses of LIF, or between untreated versus treated endometria, at either day of pregnancy, at the $p<0.05$ level.

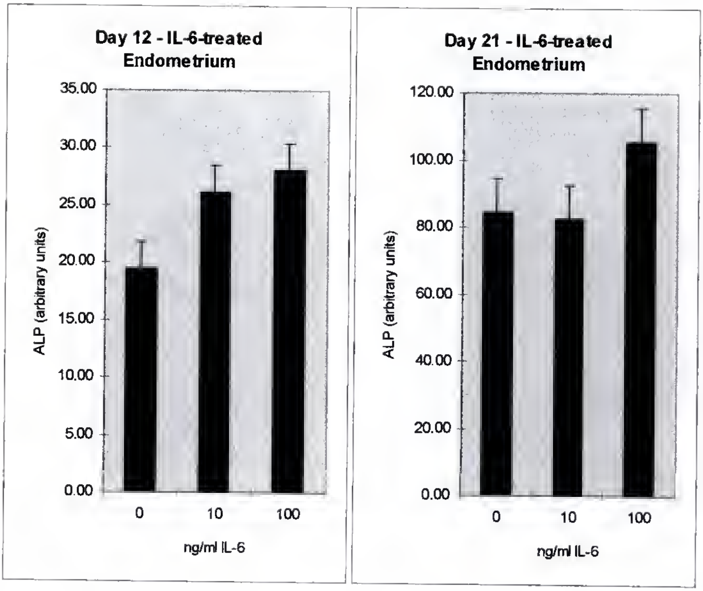


Figure 5-7. The effect of IL-6 on the level of ALP mRNA in days 12 and 21 porcine endometrial explants.

X-values are days of pregnancy; Y-values are LS means of radioactivity volumes. Error bars represent LS SEM. Each graph is a result of experiments with 3 animals, repeated 2 or 3 times each. By orthogonal contrast analysis, ALP radioactivity volume was different ($p<0.01$) in untreated day 12 explants versus the treated cultures. In day 21 explants treated with 100 ng/ml IL-6, ALP mRNA, abundance was greater than in those treated with 10 ng/ml IL-6 or controls ($p<0.03$).

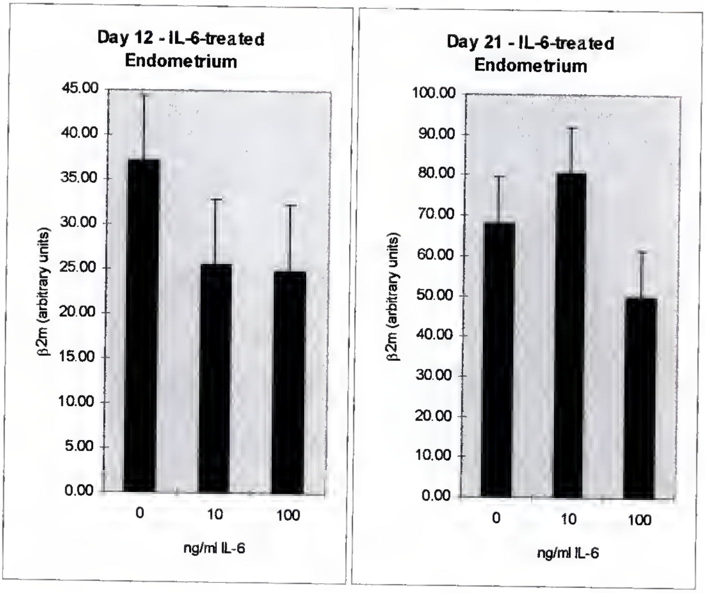


Figure 5-8. The effect of IL-6 on the level of β_2m mRNA in days 12 and 21 porcine endometrial explants.

X-values are days of pregnancy; Y-values are LS means of radioactivity volumes. Error bars represent LS SEM. Each graph is a result of experiments with 3 animals, repeated 2 or 3 times each. By orthogonal contrast analysis, β_2m radioactivity volume was significantly different in untreated day 12 explants versus the treated cultures ($p<0.02$).

Effects of Culture Time on ALP and β_2m mRNA abundance

The radioactivity volumes of ALP- and β_2m-probed dot-blots were compared with regard to the duration of culture. Table 5-1 is the ANOVA table of results from the cytokine-untreated endometria, cultured for 0, 6 and 24 hours, from which total RNA was isolated and dot-blotted with the radiolabeled probe for ALP mRNA. Table 5-2 is the ANOVA table of the same RNA, but dot-blotted with the radiolabeled probe for β_2m mRNA. Table 5-3 shows LS means, standard errors and orthogonal contrast analysis results of porcine day 12 and 21 endometrial explants cultured for 0, 6 and 24 hours and hybridized with radioactive ALP and β_2m mRNA probes. The effect of culture duration on day 12 and day 21 endometrial explant ALP mRNA abundance is shown in Figure 5-3. In day 12 endometrial explants, ALP mRNA in endometria cultured for 6 and 24 hours was increased by 40% and 58% ($p<0.02$), respectively, when compared to uncultured endometria. This effect was time-dependent, with the mRNA amount being greatest for explants cultured for 24 hours. There was no significant difference in ALP mRNA expression between cultured and uncultured day 21 endometria. In day 21 endometrium, ALP expression was 55% higher in explants cultured for 6 hours, compared to explants cultured for 24 hours ($p<0.04$). The levels of β_2m mRNA were not statistically different between cultured and fresh day 12 and 21 endometria (Fig 5-4). In day 21 endometrium, however, explants cultured for 6 hours had 32% greater levels of β_2m mRNA expression than did explants cultured for 24 hours ($p<0.02$).

The effect of LIF and IL-6 on ALP and β_2m mRNA abundance

The radioactivity volumes of ALP- and β_2m-probed dot-blots were compared with regard to the treatment, day of pregnancy, animal and interactions. Table 5-4 is the ANOVA table of results from the cytokine-treated endometria, cultured 6 and 24 hours, from which total RNA was isolated and dot-blotted with the radiolabeled probe for ALP mRNA. Table 5-5 is the ANOVA table of the same RNA, but dot-blotted with the radiolabeled probe for β_2m mRNA. Table 5-6 shows LS means, standard errors and orthogonal contrast analysis results of porcine day 12 and 21 endometrial explants treated with 0, 10 and 100 ng/ml of LIF or IL-6 and hybridized with radioactive ALP and β_2m mRNA probes.

The effect of LIF on ALP and β_2m mRNA abundance

The effects of LIF on ALP mRNA expression in endometrial explants are shown in Figure 5-5. The level of ALP mRNA was 37% higher in day 12 explants treated with LIF, than in untreated explants. By orthogonal contrast analysis, this difference was found to be significant ($p<0.01$). In day 12 endometrium, there was also a difference in ALP expression between explants treated with 10 vs. 100 ng/ml LIF ($p<0.01$). In day 21 endometria, the orthogonal contrast analysis showed that the apparent differences were not statistically significant. The effects of LIF on β_2m expression in endometrial explants are shown in Figure 5-6. In day 12 and 21 endometria, orthogonal contrast analysis showed that differences between the treated and untreated explants were not statistically significant for either group.

The effect of IL-6 on ALP and β_2m mRNA abundance

The effect of IL-6 on ALP mRNA abundance in endometrial explants is shown in Figure 5-7. In day 12 endometrium, the levels of ALP mRNA expression increased with concentration of exogenous IL-6. The difference in ALP mRNA expression between untreated explants and those treated with 10 and 100 ng/ml IL-6, was 34% and 44%, respectively ($p<0.01$). In day 21 endometrium there was no difference in ALP expression between IL-6 treated and untreated samples. However, there was a difference between the two doses ($p<0.05$). The effect of IL-6 on β_2m mRNA expression in endometrial explants is shown in Figure 5-8. In day 12 endometria, there was a negative effect observed with both concentrations of IL-6. The decrease of β_2m expression in explants treated with 10 and 100 ng/ml was 31% and 33%, respectively. These differences were significant ($p<0.02$). In day 21 endometrial explants, β_2m mRNA expression in treated samples was not different from untreated cultures. In 100 ng/ml IL-6 treated explants, β_2m mRNA was 38% lower than in the 10 ng/ml IL-6 treated explants. By orthogonal contrast analysis, this difference was found to be borderline significant ($p<0.06$).

Discussion

Northern blot hybridization demonstrated that the ALP probe detected an RNA of the expected size. Statistical analysis of RNA dot blot hybridizations of explant RNAs using the ALP probe, revealed effects of culture time, in the explants that did not receive any treatment. In untreated day 12 endometrial explants, there was a significant positive correlation between the duration of culture and ALP mRNA expression. A 50% increase in ALP expression in culture suggests that there may be a factor, produced either by conceptuses or by the mother, which suppresses ALP expression *in vivo* at this time of development. When this hypothetical factor is removed in culture over time, ALP gene expression is increased. In day 21 tissues, an opposite trend was observed, with ALP expression decreasing in culture over time. However, the difference in duration of culture in day 21 endometrium was not statistically significant. The small sample size might influence this finding; if the difference were significant, indeed, this might indicate the presence of an inducing factor, or intermediary product, *in vivo*, that is removed during the *in vitro* technique.

The treatment of explants with 100 ng/ml LIF resulted in a one-third increase in ALP mRNA expression in day 12, but not in day 21 endometrial explants. The effect of 10 ng/ml LIF on ALP expression in day 12 explants was negligible. A possible explanation for the lack of effect of the lower dose of LIF may be the lower potency of LIF; i.e., a higher effective dose is required to exhibit a physiological effect. This would be in concordance with findings of Anegon et al. (1994), who reported the detection of 13,000 U/ml LIF in day 12 porcine ULF. In our study, the concentrations of hrLIF used were 86 and 860 U/ml, which are lower than the concentrations found in day 12 ULF. A possible explanation for the absence of an effect of LIF on ALP mRNA abundance in day 21 endometrium may be that the basal expression of ALP in day 21 endometrium was already 5 times higher than in day 12 endometrium. If the regulatory region of the ALP gene was already activated by another factor, LIF could not additionally increase the already high transcription rate. Alternatively, the absence of the effect of LIF on ALP expression in day 21 explants may further support the hypothesis that the effective dose of LIF for porcine endometrium is higher than the dose used. In addition, human LIF may be less effective in the porcine model than is porcine LIF. Human recombinant LIF can induce a physiological response by porcine cartilage cells (Bell and Carroll, 1995), but its exact specific activity for porcine cells remains unknown.

The treatment of day 12 explants with 10 and 100 ng/ml (19 and 190 U/ml, respectively) of human IL-6 resulted in moderate, though significant, dose-dependent increases in ALP mRNA abundance. This suggests that hrIL-6 may be more potent than hrLIF in inducing physiological effect on porcine cells. Although the level of induction is moderate, IL-6 concentrations used were lower than those reported by Anegon et al. (1994) for days 11 and 12 pregnant porcine ULF (between 10,000 and 15,000 U/ml). In day 21 explants, only the higher dose of IL-6 induced a positive effect on ALP mRNA expression. A possible explanation, similar to that for LIF, may be that the basal expression of ALP in day 21 endometrium was already 5 times higher than in day 12 endometrium, and only the higher dose of IL-6 was potent enough to induce an effect on ALP synthesis. The positive effects of LIF and IL-6 on ALP expression are consistent with previous observations from human lung tissues, where peak expression of both ALP (Maruyama et al., 1994) and IL-6 mRNAs (Bedard et al., 1993; Crestani et al., 1994) occurs at times of infection.

There are other indications that hematopoietic cytokines may be physiological inducers of ALP expression. Sallenave et al. (1994) reported that ALP synthesis in human airway epithelial cells is induced by IL-1 and TNF-α. These same cytokines have been found to induce synthesis of LIF in astrocytes (Aloisi et al., 1994), bone marrow (Derigs and Boswell, 1993), cartilage (Lotz et al., 1992) and fibroblasts (Hamilton et al., 1993). Both of these cytokines also induce the synthesis of IL-6 in osteoblasts and human bone marrow stromal cells (Rifas et al., 1995), cultured human endothelial cells (Brown et al., 1991), synovial fibroblasts (Richards and Saklatvala, 1991) and astrocytes (Norris et al., 1994). It is not known, however, whether IL-1 and TNF-α are physiological inducers of ALP in porcine endometrium, since it is not known whether these two cytokines are present in porcine endometrium at the time of ALP gene expression. In other species, such as the mouse, IL-1 is abundantly present in the endometrium at various stages of the reproductive cycle and pregnancy (De et al., 1992; De et al., 1993; Kover et al., 1995).

HrIL-6 exhibited a significant negative effect on the expression of β_2m gene in porcine endometrium. This is in contrast to a previous finding that IL-6 induced β_2m mRNA abundance in cultured rat hepatocytes (Roncero et al., 1995). The possible reason for this difference is that IL-6 may be regulated differently between the two species, or between the two different tissues examined. Since it is known that cytokines interact with one another, it is possible that the effect of a given cytokine depends on the coexpression with other cytokines. Moreover, hematopoietic cytokines are known to induce opposing effects in different cell types. For example, LIF can induce (Williams et al., 1988) or inhibit (Rathjen et al., 1990) cell differentiation, as well as promote (Estrov et al., 1995; Kellokumpu-Lehtinen et al., 1996) or arrest (Gearing et al., 1987) malignant growth.

From the data obtained, it seems that LIF and IL-6 may be physiological modulators of ALP expression, since both genes and gene products are coexpressed in the reproductive tract during pregnancy. Although the effects of LIF and IL-6 treatment on the ALP mRNA expression in cultured endometria were statistically significant, the differences between treated and untreated endometria were only moderate. Therefore, the physiological role of these cytokines with regard to regulation of ALP gene may not be important. Nevertheless, there is a possibility that LIF and IL-6 act synergistically to induce ALP, as is the case for certain other cytokines (Hamilton et al., 1993; Mackiewicz et al., 1993). In this study, however, we did not test the combination of both cytokines. It is likely that the day preference for a particular cytokine depends on the differential endometrial expression of low-affinity receptor complexes for LIF and IL-6, i. e. LIFR-β and IL-6R-α, respectively. Unfortunately, there are no data available for IL-6R-α that could be compared with LIFR-β data. Our findings of LIFR-β expression in days 12 and 18 porcine endometrium (Chapter 4) agree with this hypothesis, assuming that the expression of LIFR-β in day 21 is similar to that on day 18. In human bronchiolar epithelial cells, ALP expression is stimulated by IL-1 and TNF-α, the two cytokines that are known inducers of LIF (Lubbert et al., 1991) and IL-6 (Norris et al., 1994; Rifas et al., 1995) expression. It is therefore possible that LIF and IL-6 are intermediary regulators of ALP expression in endometrium. ALP expression in porcine uterus may be put in a broad context as a determining factor for the invasiveness of placentation across species, with the species that exhibit less invasive implantation expressing more ALP (Badinga et al., 1994). One has yet to determine how LIF and IL-6 induction of ALP fits into this model. There is no evidence demonstrating higher LIF or IL-6 expression in endometrium of species with less invasive implantation.

In other systems, LIF and IL-6 have been associated with stimulation of synthesis of several proteinase inhibitors other than ALP. The production of TIMP, another proteinase inhibitor, was enhanced by IL-6 in a dose dependent manner in human skin and uterine cervical fibroblasts (Sato et al., 1990). Other investigators observed similar results, using OM, IL-6, LIF, and IL-1-α in concentrations of 5 to 50 ng/ml, which elevated

TIMP-1 expression at the RNA level in human fibroblasts of lung or synovial origin (Richards et al., 1993). Additional evidence that hematopoietic cytokines regulate TIMP-1 expression was given by Bugno et al. (1995), who identified an IL-6/OSM response element at -64 to -36 nucleotides, which is operative in hepatic cells. Moreover, within this element, the authors have identified the functional binding site for STAT3, which is stimulated by hematopoietic cytokines via the JAK/STAT pathway. LIF and IL-6 also stimulated synthesis of α_2m, which is an APR with proteinase inhibitor activity, in cultured astrocytes and hepatocytes in the presence of glucocorticoid (Higuchi et al., 1994). In addition, IL-6 stimulated the expression of the serine protease inhibitor 2.3 gene (spi 2.3) in cultured rat hepatocytes. The 5' flanking region of this gene binds the IL-6-inducible acute phase response factor present in liver nuclear extracts from inflamed rats (Simar-Blanchet et al., 1996).

Pig blastocysts, similar to those of other species, are invasive when placed at ectopic sites (Samuel, 1971). Since the type of placentation in swine is the least invasive of those of domestic animals (Mossman, 1987), a maternal factor may exist, that prevents the erosion of endometrium by the trophoblast. From the available literature, and the data presented here and elsewhere, it appears tbat LIF and IL-6 stimulate the synthesis of a spectrum of proteinase inhibitors. It would also be interesting to know what effects these cytokines have on expression of proteinases, for which there is minimal data available. However, there are indications that hematopoietic cytokines may inhibit the synthesis of proteinases. The study by Harvey et al. (1995) found an inhibitory effect of LIF on the mRNA expression of two proteinases, uPA and MMP-9 in cultured mouse blastocyst outgrowths. If the effect of LIF or IL-6 on the expression of other proteinases is also inhibitory, this would suggest that one general function may be to reduce the invasiveness of trophoblast *in vivo*.

CHAPTER 6
EXPRESSION OF LEUKEMIA INHIBITORY FACTOR, LEUKEMIA INHIBITORY FACTOR RECEPTOR AND INTERLEUKIN-6 GENES IN JAG-1 CELLS

Introduction

Trophoblast is the outermost layer of cells of the pig blastocyst. It is an extraembryonic ectoderm which contributes to the outer layer of the chorion. Trophoblast makes contact with the maternal tissue of the uterine wall, allowing for nourishment of the embryo proper (Mossman, 1987). Trophoblast growth is rapid in order to accommodate the growing needs of the developing embryo. Porcine trophoblast undergoes tremendous remodeling on days 11 or 12, resulting in the elongation of porcine embryos from about 10 mm to about 1 m in length, in just one day. This change is primarily a result of tissue remodeling and not of cell division (Geisert et al., 1982; Pusateri et al., 1990). It is not known what factor(s) induce elongation of pig conceptuses. The signal(s) for elongation may originate in the embryo, or from the endometrium. Conceptus aromatase and 17-α-hydroxylase (Green et al., 1995), endometrial LIF (Anegon et al., 1994) and conceptus IL-6 genes change their expression levels coincident with onset of conceptus elongation(Chapter 3). Moreover, steroidogenic enzyme mRNAs are only transiently expressed by conceptuses just prior to elongation (Green et al., 1995).

Tissue remodeling is mediated by proteinases (Strickland et al., 1976), which are themselves, inhibited by proteinase inhibitors (Werb et al., 1992). Blastocysts of various species were found to synthesize proteinases and / or their inhibitors. For example, periimplantation mouse blastocysts synthesize proteinases (Brenner et al., 1989; Behrendtsen et al., 1992; Harvey et al., 1995), while mouse (Brenner et al., 1989), sheep and bovine blastocysts (Kramer et al., 1994) were found to synthesize proteinase inhibitors. These findings suggest that conceptuses of mammalian species undergo active tissue remodeling by cell-extracellular matrix interactions, which are regulated by proteinases and their inhibitors. The mRNA expression of proteinases uPA and MMP-9 in mouse embryos is negatively regulated by LIF (Harvey et al., 1995). The mRNA expression of some proteinase inhibitors, such as rat serine protease inhibitor 2.3 (spi 2.3) is upregulated in cultured hepatocytes by IL-6 (Simar-Blanchet et al., 1996), while the expression of another proteinase inhibitor, α_2m mRNA is stimulated by both LIF and IL-6 in rat brain and liver (Higuchi et al., 1994). It is, therefore, possible that the elongation of porcine embryos is regulated in part, by hematopoietic cytokines.

It is not known whether porcine trophoblast expresses LIF or IL-6 genes and proteins. The expression of LIF and IL-6 mRNAs was detected in mouse trophoblast cells (Murray et al., 1990), while the expression of IL-6 mRNA was detected in porcine, ovine and bovine (Mathialagan et al., 1992) conceptuses, but it is unclear in what cell type(s) this expression occurs. Human trophoblast expresses IL-6 (King et al., 1995; Stephanou et al., 1995), but not LIF mRNAs (King et al., 1995). The postulated effect of LIF on human trophoblast is induction of differentiation (Arici et al., 1995; Kojima et al., 1995; Nachtigall et al., 1996), while in the mouse, is stimulation of trophoblast outgrowth (Lavranos et al., 1995) and induction of proteinase activity (Harvey et al., 1995). The possible function of LIF synthesized by porcine trophoblast may be directed toward the maternal endometrium, trophoblast, or inward, toward the ICM, since the endometrium and conceptuses express the LIFR-β gene at this developmental time (Chapter 4). Taking all of this into consideration, LIF and IL-6 may be involved in porcine trophoblast proliferation and / or ICM differentiation.

LIF exhibits its physiological effects through LIFR-β binding to gp130, and subsequent activation of the JAK/STAT pathway of gene transcription (Gearing et al., 1991; Stahl et al., 1994; Minami et al., 1996). LIFR-β is known to be expressed in human trophoblast (King et al., 1995; Kojima et al., 1995). LIFR-β biosynthesis may be regulated by LIF, since other hematopoietic cytokines can alter the expression levels of their cognate receptors (Lasfar et al., 1994; Schwabe et al., 1994). It is also possible that other factors induce the expression of LIFR-β.

Beta$_2$m is the light (β) chain of the class I MHC. In swine, this is a 119 amino acid, 11-kD protein (Milland et al., 1993). The heavy chain of mouse class I MHC is a 40- to 45-kD protein, that consists of α_1, α_2 and α_3 domains (Heise et al., 1987; Gill et al., 1987). The Beta$_2$m gene resides on a different chromosome than that for the heavy chain of the type I MHC (Flavell et al., 1986), and there are indications that β_2m expression in mouse endoderm (Jaffe et al., 1991), preimplantation human blastocysts (Jurisicova et al., 1996) and other cells (Morello et al., 1985; Israel et al., 1989), may be regulated independently from the genes encoding the α chain proteins. However, the genes encoding both the MHC light and heavy chains have NF-κB response elements in their respective regulatory regions (Israel et al., 1989), as does the IL-6 gene (Kopp and Ghosh, 1994). Beta$_2$m mRNA is not detectable in human early preimplantation blastocysts (Desoye et al., 1988), but its synthesis is initiated shortly before implantation (Jurisicova et al., 1996). In the mouse, the developmental expression of β_2m is first detectable in the extra-embryonic derivatives of the early primitive streak-stage embryo (Jaffe et al., 1990). It has been postulated that regulation of MHC gene expression during early development is critical for maternal tolerance of the fetal allograft (Desoye et al., 1988).

The Jag-1 cell line was established from culture of trophoblastic tips of day 14 porcine conceptuses (Ramsoondar et al., 1993). These cells were described as being cytokeratin-positive and vimentin-negative, and therefore of epidermal origin. Jag-1 cells share various morphological characteristics with porcine trophoblast. Like trophoblast cells from other species, these cells do not express class I or II MHC antigens, but do secrete a factor that exhibits GM-CSF activity (Ramsoondar et al., 1993). It is not known whether Jag-1 cells express hematopoietic cytokines or their receptors. By comparison, mouse blastocysts at the stage of 3.5 days post-estrus expresses IL-6 and LIF genes (Murray et al., 1990), and day 14 porcine blastocysts (Mathialagan et al., 1992) and human trophoblast (King et al., 1995; Stephanou et al., 1995) express the IL-6 gene. In addition, porcine ICM differentiation may be inhibited by LIF (Hochereau de Reviers and Perreau, 1993), indicating the presence of LIFR-β in these cells.

This study examined the hypothesis that LIF, LIFR-β, IL-6 and β_2m genes are co-expressed in JAG-1 cells and that these cells are therefore an appropriate model for studying pig trophoblast signaling molecules. The objective of this study was to monitor the expression of LIF, LR, IL-6 and β_2m mRNAs in JAG-1 cells.

Materials and Methods

Cell culture

Frozen Jag-1 cells were provided by Dr. Jagdeece Ramsoondar (Texas A&M University). Cells were thawed and resuspended in fetal bovine serum (FBS)-enriched (10%) Dulbecco's modified Eagle's medium and Ham's F12 (DME/F12, 1:1) with antibiotic-antimicotic (ABAM) (Sigma Chemical Company; St. Louis, MO). Cells were cultured in 100 mm tissue culture dishes (Corning Glassworks, Corning, NY) at 37°C in 95% air / 5% CO_2. Medium was replaced daily for the first two days, and then every other day until cells reached confluence.

RT-PCR

Total cellular RNA was isolated from three plates of Jag-1 cells by using 3 ml of TRIzol reagent (GIBCO BRL; Gaithersburg, MD) to each plate, following the protocol provided with the reagent, except that RNA was precipitated in isopropanol at -80°C overnight. PCR primers, buffers and cycling parameters for amplifying LIF, IL-6 and β_2m, were exactly as described in Chapter 3. PCR primers, buffer and cycling parameters for amplifying LIFR-β exactly as described in Chapter 4. PCR products were electrophoresed in 1.5% agarose gels and photographed over UV light. The molecular weight markers were λ/HinfI and pUC18/Sau_3AI.

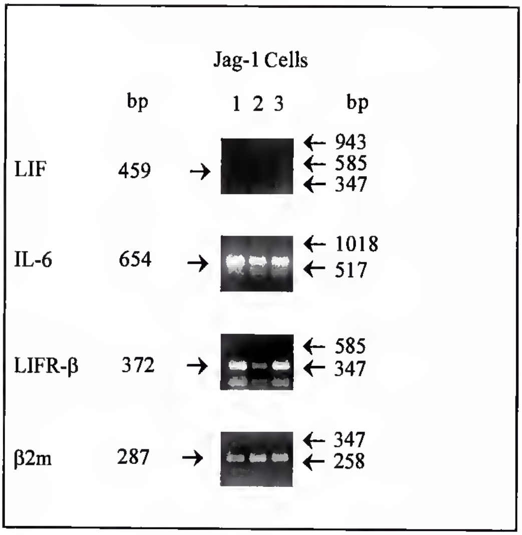


Figure 6-1. Ethidium bromide-stained agarose gels containing Jag-1 cDNAs amplified by PCR, using primers for LIF, IL-6, LIFR-β and β_2m.
The numbers of PCR cycles for obtaining LIF, IL-6, LIFR-β and β_2m products were 40, 35, 40 and 25, respectively. The numbers on the right are sizes of DNA markers (λ/HinfI for IL-6, and pUC18/Sau_3AI for LIF, LIFR-β and β_2m. Numbers represent products obtained from different culture plates.

Results

A composite picture of 1.5% agarose gels containing RT-PCR products from Jag-1 total cellular RNA, using primers for LIF, IL-6, β_2m and LIFR-β, are shown in Figure 6-1. LIFR-β, IL-6 and $\beta_2$microglobulin mRNAs were, unlike LIF, found to be expressed in cultured Jag-1 cells.

Discussion

The day 14 trophoblast cell line, Jag-1, does not express LIF mRNA in amounts that can be detected by RT-PCR, using the above-described conditions. This finding is similar to those for porcine day 11 embryos and day 18 extraembryonic membranes, as described in Chapter 3. Mouse extraembryonic tissues express LIF mRNA in both pre- and postimplantation stages (Conquet and Brulet, 1990). In contrast to mouse, but like porcine trophoblast, LIF mRNA was absent in human early pregnancy trophoblast (King et al., 1995). These data suggest that there may be species-specific differences in expression of LIF in trophoblast, as is the case for other pregnancy-related genes (Roberts et al., 1987; Badinga et al., 1994).

IL-6 mRNA was detected by RT-PCR of Jag-1 cells. This finding is in agreement with the detection of IL-6 mRNA in day 11 filamentous porcine conceptuses (Chapter 3), and with the findings of Mathialagan et al. (1992), who detected IL-6 mRNA in days 13 to 21 porcine conceptuses. In other systems, IL-6 expression is induced by IL-1 and TNF-α (Norris et al., 1994; Rifas et al., 1995), PDGF (Franchimont and Canalis, 1995), OSM (Brown et al., 1991) and IL-4 (Donnelly et al., 1993). It is not known which, if any, factors induce IL-6 in porcine trophoblast, due to the fact that expression of IL-6 inducing factors induce IL-6 in porcine trophoblast, due to the fact that expression of IL-6 inducing cytokines in porcine reproductive tract has not been studied. However, regulation of IL-6 expression has been studied extensively in the human and mouse. Ovariectomy of mice results in increased IL-6 expression in bone marrow and this can be repressed with estrogen. Estrogen inhibits the expression of the human IL-6 gene through an estrogen receptor-mediated indirect effect on transcriptional activity of the proximal 225-bp sequence of the promoter (Pottratz et al., 1994). The repression depends on the presence of both estrogen (E_2) and estrogen receptor (Jilka et al., 1995). E_2 and ER inhibit NF-IL-6 and the p65 subunit of NF-κB binding to the IL-6 promoter (Ray et al., 1994). Nonetheless, there is an antithetical report that significant amounts of IL-6 mRNA appeared in uterus following the exposure of ovariectomized mice to E_2 and P_4 (De et al., 1992). It appears that the absence of estrogen may be a positive factor for IL-6 gene expression in porcine trophoblast. Taken together with the finding that filamentous porcine conceptuses express aromatase at low or undetectable levels (Green et al., 1995) and that Jag-1 cells do not express aromatase mRNA (Green, personal communication), the absence of estrogen is in temporal agreement with the high expression of IL-6 mRNA in Jag-1 cells.

We can only speculate as to the role of IL-6 in pregnancy (Tabibzadeh et al., 1995). If IL-6 plays a role in pregnancy, it is not an essential one, since mice defective in the expression of IL-6 develop normally (Kopf et al., 1994). However, taking into consideration the reports that IL-6 stimulates local inflammation (Dinarello, 1992; Bradding et al., 1993), and that endometrial inflammation is observed at the blastocyst attachment sites (Mathialagan et al., 1992), it is possible that IL-6 is a factor which causes inflammation of the underlying endometrium. This would be beneficial for the nutrition of the blastocyst, since inflammation may result in an increased blood flow to the implantation sites. Besides having a role in conceptus nutrition, IL-6 may also indirectly be involved in iron transport. Uteroferrin (UF) is important for transport of iron from mother to conceptus (Gaber et al., 1979). UF is one of the major proteins synthesized by pig uterine glands and the best characterized one (Dantzer and Nielsen, 1984; Simmen et al., 1991). There are indications that UF may be induced by IL-6, since an IL-6 response element was identified in the regulatory region of uteroferrin gene (Kwak, personal communication).

The detection of LIFR-β mRNA in this study is in concert with previous reports of LIF exhibiting physiological effects on human and mouse blastocysts. One effect of LIF on mouse eight-cell embryo development *in vitro* is acceleration of rates of embryo hatching and trophoblast outgrowth (Lavranos et al., 1995). The other relates to findings that LIF induces differentiation of purified cultures of human cytotrophoblasts, as indicated by decreased production of hCG mRNA by human choriocarcinoma cells (Kojima et al., 1995), and increased expression of oncofetal fibronectin mRNA and protein by human cytotrophoblast cells (Nachtigall et al., 1996). Inhibition of hCG and gelatinases in human cytotrophoblastic cells by LIF was observed by Bischof et al. (1995). In addition, embryo development to the blastocyst stage was significantly enhanced in two-cell embryos cultured with cell lines that synthesize LIF (Kauma and Matt, 1995). The second band of the smaller size then expected for LIFR-β may be an artefact resulting from non-specific primer binding, or a result of self-priming. The PCR primers have homology to the region on LIFR-β gene that encodes extracellular receptor domain, and thus detects both mRNAs for the soluble and membrane-bound receptor. Therefore, an additional band is probably not a result of differential splicing.

The above mentioned effects of LIF on trophoblast and while blastocysts are likely to be mediated by LIFR-β. This is supported by detection of LIFR-β mRNA and protein in trophoblast, ICM and whole conceptuses of various species. LIFR-β was detected in human implantation blastocysts (Charnock-Jones et al., 1994), murine ES cells (Rose et al., 1994) and human placenta (Kojima et al., 1995). LIFR-β may be a mediator of porcine conceptus remodeling because LIF has been found to induce the synthesis of the proteinases MMP-9 and uPA in early mouse embryos (Harvey et al., 1995), and these proteinases have been proposed to play a role in tissue remodeling (Sappino et al., 1989; Werb et al., 1992). It is unknown what maintains the expression of LIFR-β in Jag-1 cells, especially with the regard to the fact that LIF was not detected in these cells. There is a possibility that IL-6 induces the expression of LIFR-β, since it was demonstrated that IL-6 induces the expression of gp130 (Schooltink et al., 1992; Snyers and Content, 1992), which is structurally (Gearing et al., 1991) and evolutionarily (Shields et al., 1995) the most closely related to LIFR-β. LIF and IL-6 are highly expressed in d30 and d60 placentae, but not d18 trophoblast (Chapter 3). This study demonstrates that porcine trophoblast *in vitro* expresses LIFR-β and IL-6 as do whole embryos. This leads to the conclusion that at least a part of the contribution of these two products comes from the trophoblast.

Jag-1 cells exhibit very slow growth compared to other cell types. In our hands, the doubling time of those cells is about 7 days, compared to 2 days of human choriocarcinoma cell line (Jeg-3). Since trophoblast proliferates at a high rate *in vivo*, it is conceivable that Jag-1 trophoblast cells in culture lack factor(s) to induce their growth. Because of the fact that LIF induces trophoblast outgrowth of mouse embryos (Lavranos et al., 1995) and human placenta (Kojima et al., 1995), and that LIFR-β is expressed in Jag-1 cells, it is possible to speculate that exogenous LIF may induce growth of Jag-1 cells.

Ramsoondar et al. (1993) failed to detect class I or II MHC antigens in Jag-1 cells. In that respect, porcine trophoblast is similar to human trophoblast (Morello et al., 1985; Desoye et al., 1988). The present study demonstrated the MHC class I light chain mRNA expression in Jag-1 cells. Providing that the antibodies used by previous studies detect only heavy chain class I MHC antigens, this study provides another example that light chain class I MHC gene is regulated independently from the heavy chain genes. The differential expression of class I MHC genes in porcine trophoblast may be a result of the fact that heavy chain molecules of the class I MHC are encoded within the HLA cluster, while β_2m is separately encoded outside of the gene cluster (Flavell et al., 1986). The differential expression of light from heavy chain class I MHC genes in other animals and cell types have been previously reported (Morello et al., 1985; Israel et al., 1989; Jaffe et al., 1991; Jurisicova et al., 1996).

Jag-1 cells may be a useful tool for studying the biological effects of exogenous factors on trophoblast, especially for those investigators interested in implantation of the porcine conceptus. These cells can be used to examine the effects of cytokines on the expression of genes normally expressed in the trophoblast. It will be possible to test the effects of LIF on Jag-1 cells, since it was demonstrated that the mRNA encoding LIFR-β is expressed by these cells. However, it is not known whether gp130 is expressed in Jag-1 cells. It would be informative to monitor the expression of gp130, in order to know whether functional LIFR, consisting of both LIFR-β and gp130, can be assembled in these cells. It is likely that gp130 is synthesized by Jag-1 cells, since the expression of gp130 mRNA is stimulated by IL-6 (Schooltink et al., 1992; Snyers and Content, 1992), whose mRNA was detected in Jag-1 cells (this study). In conclusion, Jag-1 cells might constitute a useful *in vitro* system for studying the regulation of these two genes' expression in trophoblast.

CHAPTER 7
SUMMARY AND CONCLUSIONS

The presented studies were undertaken to document the developmental expression of the hematopoietic cytokines, LIF and IL-6, and to examine their effects on the pig endometrium. First, the temporal and spatial expression of both cytokines in the swine reproductive tract was examined, as described in Chapter 3. Using the RT-PCR technique, the expression of mRNAs for LIF and IL-6 was examined in days 11, 18, 30 and 60 porcine conceptuses or placentas and in days 0, 11, 18, 30 and 60 pregnant endometria. It was demonstrated that LIF mRNA was abundantly expressed in day 30 endometrium and placenta, while at the period preceding implantation, its expression in endometrium and conceptuses was at or below the limits of detection, respectively. These data suggest that there may be a preferential role for LIF at the period after implantation. The finding of low levels of LIF mRNA in preimplantation porcine endometria is in contrast to the previously reported data from human and mouse preimplantation endometria. A comparative study of LIF expression in preimplantation endometria has not been performed, but it is likely that the abundance of endometrial LIF mRNA at the time of implantation is in correlation with the type of placentation, where LIF would have a physiological role in species exhibiting decidualization.

IL-6 mRNA was expressed in both endometria and conceptuses during the period before implantation, but more abundantly after implantation, indicating a possible greater role of this cytokine after implantation is completed. Interestingly, the expression of IL-6 mRNA was detected in all litters consisting of filamentous, but not spherical conceptuses. In these two developmentally close morphologies, there are differences with regard to the secretion of signaling molecules, most notably in the level of estrogen, which is abundantly synthesized in spherical, but much less abundantly in filamentous, conceptuses. Knowing that estrogen is a suppressor of IL-6 in other systems, it is postulated that estrogen suppresses IL-6 synthesis in porcine conceptuses at the transcription level.

Beta$_2$m mRNA was uniformly distributed among all endometrial specimens examined, and placental specimens, starting from day 18. However, the levels of β_2m mRNA in day 11 conceptuses varied between pools of litters. Together with previously documented data from human and mouse, it suggests that β_2m expression is initiated around the time of implantation.

The presented studies examined temporal and spatial expression of the cytokine-binding subunit of the LIF receptor, LIFR-β mRNA in the swine reproductive tract by RT-PCR. Expression of LIFR-β mRNAs was examined in days 11, 18, 30 and 60 porcine conceptuses or placentas (Chapter 4), in day 14 porcine trophoblast cells (Chapter 6), in days 0, 11, 18, 30 and 60 pregnant endometria and in day 12 porcine endometrial primary cell cultures (Chapter 4). It was demonstrated that LIFR-β mRNA was expressed in both pre- and postimplantation porcine endometria and conceptuses, but more abundantly in day 30 endometrium and placenta, suggesting that LIFR-β may mediate the effect of

Table 7-I. Summary of RT-PCR amplification of LIF, LIFR-β, IL-6 and β_2m mRNA in porcine conceptuses, placentae, endometrium, and endometrial cell cultures.

Gene	**Jag-1**	**Conceptus**		**Placenta**			**Endometrium**						**Endometrial cells**		
		d11S	d11F	d18	d30	d60	d0	d11S	d11F	d18	d30	d60	ST	LE	GE
LIF	-	+/-	-	+/-	++	++	+/-	+	+/-	-/+	+++	-	-	-	-
LIFR-β	++	+	+	-	++	++	+/-	++	+/-	+	+++	+	++	++	++
IL-6	+++	-	++	++	+++	+++	++	+	+	-	+++	+++	++	+	++
β_2m	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++

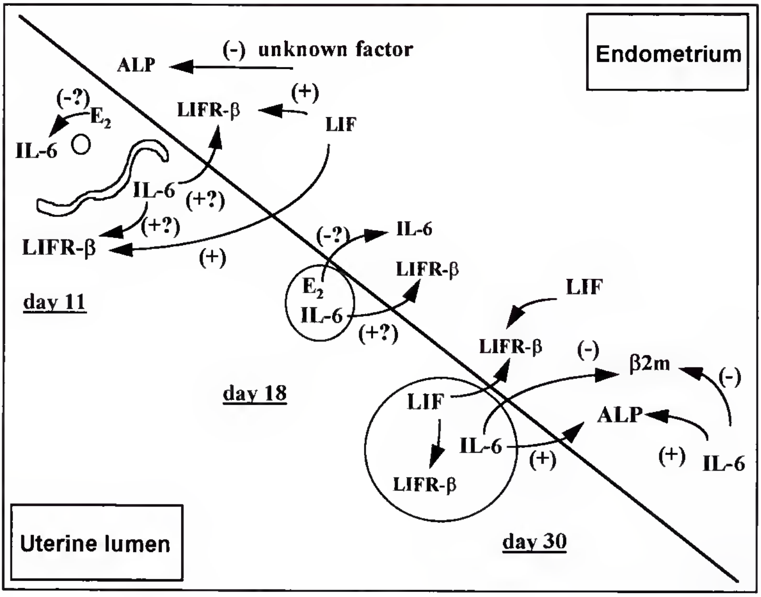


Figure 7-1. Proposed physiological model of the action of LIF and IL-6 in the porcine uterus during implantation, based on results reported in this dissertation.

hematopoietic cytokines on conceptus and endometrial cells during these stages. While LIF mRNA was most abundant in day 30 placenta, LIFR-β mRNA expression was the highest in day 30 endometrium, suggesting feto-maternal communication using LIF as a molecular signal. It is not known whether LIFR-β is expressed by porcine ICM, but the obtained data demonstrated that LIFR-β is expressed in day 14 porcine trophoblast. Beta$_2$m, the light chain class I MHC mRNA was detected in Jag-1 cells, in contrast to the previous reports of the absence of class I MHC antigens in these cells, indicating that the genes encoding light and heavy chains of class I MHC are independently expressed in porcine day 14 trophoblast.

LIF and IL-6 have been found to either stimulate or inhibit genes encoding proteinases or proteinase inhibitors, mostly stimulating proteinase inhibitor genes, while inhibiting proteinase genes. ALP is a molecule expressed more abundantly in species with epitheliochorial placentation and is proposed to protect endometrium from the erosion caused by conceptus proteinases. Therefore, hematopoietic cytokines, if expressed during porcine pregnancy, may regulate tissue remodeling of either endometrium or conceptuses, or both. The study presents data on the *in vitro* effects of LIF and IL-6 on the expression of ALP and β$_2$m mRNAs in pregnant porcine endometrium (Chapter 5). Both cytokines moderately stimulated the expression of ALP mRNA in day 12 endometrial explants, suggesting that the post-implantation role of these cytokines may be to determine the degree of endometrial erosion by the conceptus proteinases.

This study raises new questions to be answered in the future. These are: what stimulates LIFR-β expression in the porcine trophoblast; are there other ligands that bind to LIFR-β in porcine trophoblast and endometrium; is there any synergy in the effects of

LIF and IL-6 on the expression of ALP and other genes; are there other proteinases regulated by LIF or IL-6, that are important for porcine placentation; would there be any changes in the microscopic structure of feto-placental interface in LIF and/or IL-6-deficient pig; would there be any changes in the course of pregnancy in such pigs?

The data obtained from this research may help further understanding of the signaling pathways that mediate the interactions of the pig blastocyst and uterus. In the long run, such knowledge may lead to new strategies to reduce embryonic mortality, and thereby, improve reproductive efficiency.

LIST OF ABBREVIATIONS

α_2m	- alpha$_2$macroglobulin
β_2m	- beta$_2$microglobulin
APR	- acute phase reaction
APRE	- acute phase response element
APRF	- acute phase responsive factor
CDF	- cholinergic neuronal differentiation factor (LIF)
cDNA	- complementary DNA
CNTF	- ciliary-neurotropic factor
CT-1	- cardiotrophin-1
D-factor	- differentiation-inducing factor (LIF)
DNA	- deoxyribonucleic acid
E_2	- estradiol
EGF	- epidermal growth factor
ESC	- embryonic stem cells
EPO	- erythropoietin
ER	- estrogen receptor
FBS	- fetal bovine serum
G-CSF	- granulocyte colony stimulating factor
GM-CSF	- granulocyte-macrophage colony stimulating factor
GH	- growth hormone (same as STH)
GHRF	- gonadotropic hormone releasing factor
gp130	- glycoprotein 130 (same as IL-6R-β)
gp190	- glycoprotein 190 (same as LIFR-β)
gp80	- glycoprotein 80 (same as IL-6R-α)
hCG	- human chorionic gonadotropin
HSF-III	- hepatocyte-stimulating factor (LIF)
ICE	- interleukin-1-beta (IL-1-β) converting enzyme
ICM	- inner cell mass
IGF	- insulin-like growth factor
IGF-BP	- IGF binding protein
IL	- interleukin
IL-6R-α	- IL-6 receptor α (same as gp 80)
IL-6R-β	- IL-6 receptor β (same as gp130)
JAK	- Janus kinase
LIF	- leukemia inhibitory factor
LIFR-β	- LIF receptor beta (same as gp190)

MEM - minimal essential medium
MHC - major histocompatibility complex
MMP-9 - matrix metalloproteinase gelatinase B
mRNA - messenger RNA
NF-IL-6 - nuclear factor-interleukin-6
OSM - oncostatin-M
p. e. - post estrus
P_4 - progesterone
PBS - phosphate-buffered saline
PCR - polymerase chain reaction
PDGF - platelet-derived growth factor
PMA - phorbol myristate acetate
POMC - proopiomelanocortin
RNA - ribonucleic acid
RT - reverse transcription
SLPI - secretory leukocyte proteinase inhibitor
STAT - signal transducer and activator of transcription
STH - somatotropic hormone (same as GH)
TGF-β - transforming growth factor-beta
TIMP 1-3 - tissue inhibitors of metalloproteinases 1 to 3
TNF-α - tumor necrosis factor-alpha
TYK - tyrosine kinase
ULF - uterine luminal fluid
uPA - urokinase-type plasminogen activator
VEGF - vascular endothelial growth factor

REFERENCES

Abdel Motal UM, Zhou MX, Siddiqi AR, Jondal M (1993) Regulation of MHC class I membrane expression by beta 2-microglobulin. Scand J Immunol 38: 395-400

Abrahamsohn PA, Zorn TM (1993) Implantation and decidualization in rodents. J Exp Zool 266: 603-28

Ace CI, Okulicz WC (1995) Differential gene regulation by estrogen and progesterone in the primate endometrium. Mol Cell Endocrinol 115: 95-103

Akita S, Webster J, Ren SG, Takino H, Said J, Zand O, Melmed S (1995) Human and murine pituitary expression of leukemia inhibitory factor. Novel intrapituitary regulation of adrenocorticotropin hormone synthesis and secretion. J. Clin. Invest. 95: 1288-98

Alexander CM, Hansell EJ, Behrendtsen O, Flannery ML, Kishnani NS, Hawkes SP, Werb Z (1996) Expression and function of matrix metalloproteinases and their inhibitors at the maternal-embryonic boundary during mouse embryo implantation. Development 122: 1723-36

Aloisi F, Rosa S, Testa U, Bonsi P, Russo G, Peschle C, Levi G (1994) Regulation of leukemia inhibitory factor synthesis in cultured human astrocytes. J Immunol 152: 5022-31

Andreani CL, Payne DW, Packman JN, Resnick CE, Hurwitz A, Adashi EY (1991) Cytokine-mediated regulation of ovarian function. Tumor necrosis factor alpha inhibits gonadotropin-supported ovarian androgen biosynthesis. J Biol Chem 266:6761-6

Andus T, Geiger T, Klapproth J, Kunz D, Heisig M, Castell J, Heinrich PC (1988) Regulation of alpha 2-macroglobulin gene expression by interleukin-6 (BSF-2/HSF). Tokai J Exp Clin Med 13: 265-76

Anegon I, Cuturi MC, Godard A, Moreau M, Terqui M, Martinat-Botte F, Soulillou JP (1994) Presence of leukaemia inhibitory factor and interleukin 6 in porcine uterine secretions prior to conceptus attachment. Cytokine 6: 493-9

Arici A, Engin O, Attar E, Olive DL (1995) Modulation of leukemia inhibitory factor gene expression and protein biosynthesis in human endometrium. J Clin Endocrinol Metab 80: 1908-15

Arzt E, Buric R, Stelzer G, Stalla J, Sauer J, Renner U, Stalla GK (1993) Interleukin involvement in anterior pituitary cell growth regulation: effects of IL-2 and IL-6. Endocrinology 132: 459-67

Badinga L, Michel FJ, Fields MJ, Sharp DC, Simmen RC (1994) Pregnancy-associated endometrial expression of antileukoproteinase gene is correlated with epitheliochorial placentation. Mol Reprod Dev 38: 357-63

Baird PN, D'Andrea RJ, Goodall GJ (1995) Cytokine receptor genes: structure, chromosomal location, and involvement in human disease. Leu Lymphoma 18: 373-83

Baniahmad A, Tsai M.J (1993) Mechanisms of transcriptional activation by steroid hormone receptors. J Cell Biochem 51: 151-6

Barth R, Afting EG (1984) Cathepsin D from pig myometrium. Characterization of the proteinase. Biochem J 219: 899-904

Baumann H, Marinkovic-Pajovic S, Won KA, Jones VE, Campos SP, Jahreis GP, Morella KK (1992) The action of interleukin 6 and leukaemia inhibitory factor on liver cells. Ciba Found Symp 167: 100-14

Bazan JF (1991) Neuropoietic cytokines in the hematopoietic fold. Neuron 7: 197-208

Bazer FW (1992) Mediators of maternal recognition of pregnancy in mammals. Proc Soc Exp Biol Med 199: 373-84

Bazer FW, Simmen RC, Simmen FA (1991) Comparative aspects of conceptus signals for maternal recognition of pregnancy. Ann N Y Acad Sci 622: 202-11

Bazer FW, Thatcher WW (1977) Theory of maternal recognition of pregnancy in swine based on estrogen controlled endocrine versus exocrine secretion of prostaglandin F2alpha by the uterine endometrium. Prostaglandins 14: 397-400

Beck CA, Garner CW (1992) Stimulation of DNA synthesis in rat uterine cells by growth factors and uterine extracts. Mol Cell Endocrinol 84: 109-18

Bedard M, McClure CD, Schiller NL, Francoeur C, Cantin A, Denis M (1993) Release of interleukin-8, interleukin-6, and colony-stimulating factors by upper airway epithelial cells: implications for cystic fibrosis. Am J Respir Cell Mol Biol 9: 455-62

Behrendtsen O, Alexander CM, Werb Z (1992) Metalloproteinases mediate extracellular matrix degradation by cells from mouse blastocyst outgrowths. Development 114: 447-56

Bell MC, Caroll GJ (1995) Leukaemia inhibitory factor (LIF) suppresses proteoglycan synthesis in porcine and caprine cartilage explants. Cytokine 7: 137-41

Bhatt H, Brunet LJ, Stewart CL (1991) Uterine expression of leukemia inhibitory factor coincides with the onset of blastocyst implantation. Proc Natl Acad Sci USA 88: 11408-12

Bicsak TA, Michelson DS, Hsueh AJ (1989) Rat granulosa cells produce a novel trypsin-like protease in response to gonadotropin treatment. Biochem Biophys Res Commun 165: 624-30

Bischof P, Haenggeli L, Campana A (1995) Effect of leukemia inhibitory factor on human cytotrophoblast differentiation along the invasive pathway. Am J Reprod Immunol 34: 225-30

Bjorkman PJ, Saper MA, Samraoui B, Bennett WS, Strominger JL, Wiley DC (1987) The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. Nature 329: 512-8

Blair RM, Geisert RD, Zavy MT, Yellin T, Fulton RW, Short EC (1991) Endometrial surface and secretory alterations associated with embryonic mortality in gilts administered estradiol valerate on days 9 and 10 of gestation. Biol Reprod 44: 1063-79

Brach MA, Riedel D, Mertelsmann R, Herrmann F (1990) Induction of monocytic differentiation and modulation of the expression of c-fos, c-fms and c-myc protooncogenes in human monoblasts by cytokines and phorbolester. Virchows Arch B Cell Pathol Include Mol Pathol 59: 54-8

Bradding P, Feather IH, Wilson S, Bardin PG, Heusser CH, Holgate ST, Howarth PH (1993) Immunolocalization of cytokines in the nasal mucosa of normal and perennial rhinitic subjects. The mast cell as a source of IL-4, IL-5, and IL-6 in human allergic mucosal inflammation. J Immunol 151: 3853-65

Brenner CA, Adler RR, Rappolee DA, Pedersen RA, Werb Z (1989) Genes for extracellular-matrix-degrading metalloproteinases and their inhibitor, TIMP, are expressed during early mammalian development. Genes Dev 3: 848-59

Brisson C, Ethier JF, Lussier JG, Goff AK, Silversider DW (1996) Bovine leukemia inhibitory factor (bLIF) is expressed in reproductive tissues including endometrial cells lines. Biol Reprod 54 (Supl 1): 297

Brown TJ, Rowe JM, Liu JW, Shoyab M (1991) Regulation of IL-6 expression by oncostatin M. J Immunol 147: 2175-80

Bugno M, Graeve L, Gatsios P, Koj A, Heinrich PC, Travis J, Kordula T (1995) Identification of the interleukin-6/oncostatin M response element in the rat tissue inhibitor of metalloproteinases-1 (TIMP-1) promoter. Nucleic Acids Res. 23: 5041-7

Bussolino F, Arese M, Silvestro L, Soldi R, Benfenati E, Sanavio F, Aglietta M, Bosia A, Camussi G (1994) Involvement of a serine protease in the synthesis of platelet-activating factor by endothelial cells stimulated by tumor necrosis factor-alpha or interleukin-1 alpha. Eur J Immunol 24: 3131-3139

Carter DA (1995) Leukaemia inhibitory factor expression in cultured rat anterior pituitary is regulated by glucocorticoids. J Neuroendocrinol 7: 623-8

Centrella M, McCarthy TL, Canalis E (1989) Beta 2-microglobulin enhances insulin-like growth factor I receptor levels and synthesis in bone cell cultures. J Biol Chem 264: 18268-71

Charnock-Jones DS, Sharkey AM, Fenwick P, Smith SK (1994) Leukaemia inhibitory factor mRNA concentration peaks in human endometrium at the time of implantation and the blastocyst contains mRNA for the receptor at this time. J Reprod Fertil 101: 421-6

Chevalier S, Fourcin M, Robledo O, Wijdenes J, Pouplard-Barthelaix A, Gascan H (1996) Interleukin-6 family of cytokines induced activation of different functional sites expressed by gp130 transducing protein. J Biol Chem 271: 14764-72

Choi I, Simmen RC, Simmen FA (1996) Molecular cloning of cytochrome P450 aromatase complementary deoxyribonucleic acid from periimplantation porcine and equine blastocysts identifies multiple novel 5'-untranslated exons expressed in embryos, endometrium, and placenta. Endocrinology 137: 1457-67

Choudhuri R, Wood GW (1993) Production of interleukin-1, interleukin-6, and tumor necrosis factor alpha in the uterus of pseudopregnant mice. Biol Reprod 49: 596-603

Coffer P, Lutticken C, van Puijenbroek A, Klop de Jonge M, Horn F, Kruijer W (1995) Transcriptional regulation of the junB promoter: analysis of STAT-mediated signal transduction. Oncogene 10: 985-94

Cohen T, Nahari D, Cerem LW, Neufeld G, Levi BZ (1996) Interleukin 6 induces the expression of vascular endothelial growth factor. J Biol Chem 271: 736-41

Conquet F, Brulet P (1990) Developmental expression of myeloid leukemia inhibitory factor gene in preimplantation blastocysts and in extraembryonic tissue of mouse embryos. Mol Cell Biol 10: 3801-5

Crestani B, Cornillet P, Dehoux M, Rolland C, Guenounou M, Aubier M (1994) Alveolar type II epithelial cells produce interleukin-6 in vitro and in vivo. Regulation by alveolar macrophage secretory products. J. Clin. Invest. 94: 731-40

Croft SM, Mortensen RF, Gewurz H (1976) Binding of C-reactive protein to antigen-induced but not mitogen-induced T lymphoblasts. Science 193: 685-7

Croy BA, Guilbert LJ,. Browne MA, Gough NM, Stinchcomb DT, Reed N, Wegmann TG (1991) Characterization of cytokine production by the metrial gland and granulated metrial gland cells. J Reprod Immunol 19: 149-66

Cullinan EB, Abbondanzo SJ, Anderson PS, Pollard JW, Lessey BA, Stewart CL (1996) Leukemia inhibitory factor (LIF) and LIF receptor expression in human endometrium suggests a potential autocrine/paracrine function in regulating embryo implantation. Proc Natl Acad Sci USA 93: 3115-20

Dantzer V (1985) Electron microscopy of the initial stages of placentation in the pig. Anat Embryol (Berl) 172: 281-93

Dantzer V, Nielsen MH (1984) Intracellular pathways of native iron in the maternal part of the porcine placenta. Eur J Cell Biol 34: 103-9

Davis S, Aldrich TH, Stahl N, Pan L, Taga T, Kishimoto T, Ip NY, Yancopoulos GD (1993) LIFR beta and gp130 as heterodimerizing signal transducers of the tripartite CNTF receptor. Science 260: 1805-8

De M, Sanford TR, Wood GW (1992) Interleukin-1, interleukin-6, and tumor necrosis factor alpha are produced in the mouse uterus during the estrous cycle and are induced by estrogen and progesterone. Dev Biol 151: 297-305

De M, Sanford TR, Wood GW (1993) Expression of interleukin 1, interleukin 6 and tumour necrosis factor alpha in mouse uterus during the peri-implantation period of pregnancy. J Reprod Fertil 97: 83-89

De Miguel MP, De Boer-Brouwer M, Paniagua R, van den Hurk R, De Rooij DG, Van Dissel-Emiliani FM (1996) Leukemia inhibitory factor and ciliary neurotropic factor promote the survival of Sertoli cells and gonocytes in coculture system. Endocrinology 137: 1885-93

Denhardt DT, Feng B, Edwards DR, Cocuzzi ET, Malyankar UM (1993) Tissue inhibitor of metalloproteinases (TIMP, aka EPA): structure, control of expression and biological functions. Pharmacol Ther 59: 329-41

Deniz G, Christmas SE, Johnson PM (1996) Soluble mediators and cytokines produced by human CD3- leucocyte clones from decidualized endometrium. Immunology 87: 92-98

Derigs HG, Boswell HS (1993) LIF mRNA expression is transcriptionally regulated in murine bone marrow stromal cells. Leukemia 7: 630-4

Desoye G, Dohr GA, Motter W, Winter R, Urdl W, Pusch H, Uchanska-Ziegler B, Ziegler A (1988) Lack of HLA class I and class II antigens on human preimplantation embryos. J Immunol 140: 4157-9

Dinarello CA (1992) Reduction of inflammation by decreasing production of interleukin-1 or by specific receptor antagonism. Int J Tissue React 14: 65-75

Dittrich E, Haft CR, Muys L, Heinrich PC, Graeve L (1996) A di-leucine motif and an upstream serine in the interleukin-6 (IL-6) signal transducer gp130 mediate ligand-induced endocytosis and down-regulation of the IL-6 receptor. J Biol Chem 271: 5487-94

Dokter WHA, Koopmans SB, Vellenga E (1996) Effects of IL-10 and IL-4 on LPS-induced transcription factors (AP-1, NF-IL6 and NF-kappa B) which are involved in IL-6 regulation. Leukemia 10: 1308-16

Donnelly RP, Crofford LJ, Freeman SL, Buras J, Remmers E, Wilder RL, Fenton MJ (1993) Tissue-specific regulation of IL-6 production by IL-4. Differential effects of IL-4 on nuclear factor-kappa B activity in monocytes and fibroblasts J Immunol 151: 5603-12

Dudley DJ, Hatasaka HH, Branch DW, Hammond E, Mitchell MD (1992) A human endometrial explant system: validation and potential applications. Am J Obstet Gynecol 167: 1774-80

Dugast I, Jegou B (1994) [Cytokines and Sertoli cell and germ cell interactions] Contracept Fertil Sex 22: 631-4

Dukas K, Sarfati P, Vaysse N, Pradayrol L (1993) Quantitation of changes in the expression of multiple genes by simultaneous polymerase chain reaction. Anal Biochem 215: 66-72

Duncan MR, Berman B (1991) Stimulation of collagen and glycosaminoglycan production in cultured human adult dermal fibroblasts by recombinant human interleukin 6. J Invest Dermatol 97: 686-92

Dusetti NJ, Ortiz EM, Mallo GV, Dagorn JC, Iovanna JL (1995) Pancreatitis-associated protein 1 (PAP 1), an acute phase protein induced by cytokines. Identification of two functional interleukin-6 response elements in the rat PAP 1 promoter region. J Biol Chem 270: 22417-21

Eisenberg SP, Hale KK, Heimdal P, Thompson RC (1990) Location of the protease-inhibitory region of secretory leukocyte protease inhibitor. J Biol Chem 265: 7976-81

Eldridge RK, Fields PA (1985) Rabbit placental relaxin: purification and immunohistochemical localization. Endocrinology 117: 2512-9

Enokihara H Nakamura Y, Nagashima S, Furusawa S, Shishido H (1994) Regulation of interleukin-5 production by interleukin-4, interferon-alpha, transforming growth factor-beta and interleukin-6. Int Arch Allergy Immunol 104 Suppl 1: 44-5

Estrov Z, Samal B, Lapushin R, Kellokumpu-Lehtinen P, Sahin AA, Kurzrock R, Talpaz M, Aggarwal BB (1995) Leukemia inhibitory factor binds to human breast cancer cells and stimulates their proliferation. J Interferon Cytokine Res 15: 905-13

Evans DB, Thavarajah M, Kanis JA (1991) Immunoreactivity and proliferative actions of beta 2 microglobulin on human bone-derived cells in vitro. Biochem Biophys Res Commun 175: 795-803

Fairchild DL, Pate JL (1991) Modulation of bovine luteal cell synthetic capacity by interferon-gamma. Biol Reprod 44: 357-63

Farmer SJ, Fliss AE, Simmen RC (1990) Complementary DNA cloning and regulation of expression of the messenger RNA encoding a pregnancy-associated porcine uterine protein related to human antileukoproteinase. Mol Endocrinol 4: 1095-104

Fishman P, Falach-Vaknine E, Zigelman R, Bakimer R, Sredni B, Djaldetti M, Shoenfeld Y (1993) Prevention of fetal loss in experimental antiphospholipid syndrome by in vivo administration of recombinant interleukin-3. J Clin Invest 91: 1834-7

Flavell RA, Allen H, Burkly LC, Sherman DH, Waneck GL, Widera G (1986) Molecular biology of the H-2 histocompatibility complex. Science 233: 437-43

Franchimont N, Canalis E (1995) Platelet-derived growth factor stimulates the synthesis of interleukin-6 in cells of the osteoblast lineage Endocrinology 136: 5469-75

Fraser CM, Mays A, Amstutz HE, Archibald J, Armour J, Blood DC, Newberne PM, Snoeyenbos GH, Huebner RA (eds.) (1986) The Merck Veterinary Manual, 6th ed. Merck & Co, Inc. Rahway, New Jersey

Fujitani Y, Nakajima K, Kojima H, Nakae K, Takeda T, Hirano T (1994) Transcriptional activation of the IL-6 response element in the junB promoter is mediated by multiple Stat family proteins. Biochem Biophys Res Commun 202: 1181-7

Fukuoka M Yasuda K, Fujiwara H, Kanzaki H, Mori T (1992) Interactions between interferon gamma, tumour necrosis factor alpha, and interleukin-1 in modulating progesterone and oestradiol production by human luteinized granulosa cells in culture. Hum Reprod 7: 1361-4.

Gaber BP, Sheridan JP, Bazer FW, Roberts RM (1979) Resonance Raman scattering from uteroferrin, the purple glycoprotein of the porcine uterus. J Biol Chem 254: 8340-2

Gadient RA, Otten U (1993) Differential expression of interleukin-6 (IL-6) and interleukin-6 receptor (IL-6R) mRNAs in rat hypothalamus. Neurosci Lett 153: 13-16

Gaddy-Kurten D, Hickey GJ, Fey GH, Gauldie J, Richards JS (1989) Hormonal regulation and tissue-specific localization of alpha 2-macroglobulin in rat ovarian follicles and corpora lutea. Endocrinology 125: 2985-95

Gearing DP, Gough NM, King JA, Hilton DJ, Nicola NA, Simpson RJ, Nice EC, Kelso A, Metcalf D (1987) Molecular cloning and expression of cDNA encoding a murine myeloid leukaemia inhibitory factor (LIF). EMBO J 6: 3995-4002

Gearing DP, Thut CJ, VandeBos T, Gimpel SD, Delaney PB, King J, Price V, Cosman D, Beckmann MP (1991) Leukemia inhibitory factor receptor is structurally related to the IL-6 signal transducer, gp130. EMBO J 10: 2839-48

Geisert RD, Brookbank JW, Roberts RM, Bazer FW (1982) Establishment of pregnancy in the pig: II. Cellular remodeling of the porcine blastocyst during elongation on day 12 of pregnancy. Biol Reprod 27: 941-55

Geisterfer M, Richards CD, Gauldie J (1995) Cytokines oncostatin M and interleukin 1 regulate the expression of the IL-6 receptor (gp80, gp130). Cytokine 7: 503-9

Geng Y, Blanco FJ, Cornelisson M, Lotz M (1995) Regulation of cyclooxygenase-2 expression in normal human articular chondrocytes. J Immunol 155: 796-801

Gill TJ, Kunz HW, Misra DN, Hassett AL (1987) The major histocompatibility complex of the rat. Transplantation 43: 773-85

Gorski J, Gannon F (1976) Current models of steroid hormone action: a critique. Annu Rev Physiol 38: 425-50

Gough NM, Gearing DP, King JA, Willson TA, Hilton DJ, Nicola NA, Metcalf D (1988) Molecular cloning and expression of the human homologue of the murine gene encoding myeloid leukemia-inhibitory factor. Proc Natl Acad Sci USA 85: 2623-7

Gough NM, Wilson TA, Stahl J, Brown MA (1992) Molecular biology of the leukaemia inhibitory factor gene. Ciba Found. Symp. 167: 24-38

Governa M, Biguzzi S (1976) Beta2-microglobulin distribution in human normal tissues. Eur J Immunol 6: 830-2

Graham CH, Connelly I, MacDougall JR, Kerbel RS, Stetler-Stevenson WG, Lala PK (1994) Resistance of malignant trophoblast cells to both the anti-proliferative and anti-invasive effects of transforming growth factor-beta. Exp Cell Res 214: 93-9

Graham CH, Lala PK (1992) Mechanisms of placental invasion of the uterus and their control. Biochem Cell Biol 70: 867-74

Green ML, Simmen, RCM, Simmen, FA (1995) Developmental regulation of steroidogenic enzyme gene expression in the periimplantation porcine conceptus: a paracrine role for insulin-like growth factor-I. Endocrinology 136: 3961-70

Gross V, Zhang B, Geng Y, Villiger PM, Lotz M (1993) Regulation of interleukin-6 (IL-6) expression: evidence for a tissue-specific role of protein kinase C. J Clin Immunol 13: 310-20

Grutter MG, Fendrich G, Huber R, Bode W (1988) The 2.5 A X-ray crystal structure of the acid-stable proteinase inhibitor from human mucous secretions analysed in its complex with bovine alpha-chymotrypsin. EMBO J 7: 345-51

Guilbert L, Robertson SA, Wegmann TG (1993) The trophoblast as an integral component of a macrophage-cytokine network. Immunol Cell Biol 71 (Pt 1): 49-57

Gussow D, Rein R, Ginjaar I., Hochstenbach F, Seemann G, Kottman A, Ploegh HL (1987) The human beta-2-microglobulin gene. Primary structure and definition of the transcriptional unit. J Immunol 139: 3132-8

Hafez ESE (ed.) (1987) Reproduction in Farm Animals, 5th ed. Lea & Febiger, Philadelphia, Pensilvania

Haimovici F, Hill JA, Anderson DJ (1991) The effects of soluble products of activated lymphocytes and macrophages on blastocyst implantation events in vitro. Biol Reprod 44: 69-75

Hales DB (1992) Interleukin-1 inhibits Leydig cell steroidogenesis primarily by decreasing 17 alpha-hydroxylase / C17-20 lyase cytochrome P450 expression. Endocrinology 131: 2165-72

Hamilton JA, Waring PM, Filonzi EL (1993) Induction of leukemia inhibitory factor in human synovial fibroblasts by IL-1 and tumor necrosis factor-alpha. J Immunol 150: 1496-502

Hanukoglu I (1992) Steroidogenic enzymes: structure, function, and role in regulation of steroid hormone biosynthesis. J Steroid Biochem 43: 779-804

Harney JP, Ali M, Vedeckis WV, Bazer FW (1994) Porcine conceptus and endometrial retinoid-binding proteins. Reprod Fertil Dev; 6: 211-9

Harvey MB, Leco KJ, Arcellana-Panlilio MY, Zhang X, Edwards DR, Schultz GA (1995) Proteinase expression in early mouse embryos is regulated by leukaemia inhibitory factor and epidermal growth factor. Development 121: 1005-1014

Heim MH (1996) The Jak-STAT pathway: specific signal transduction from the cell membrane to the nucleus. Eur J Clin Invest 26: 1-12

Heinzel R, Appelhans H, Gassen G, Seemuller U, Maschleidt W, Fritz H, Steffens G (1986) Molecular cloning and expression of cDNA for human antileukoprotease from cerix uterus. Eur J Biochem 160: 61-7

Heise ER, Cook DJ, Schepart BS, Manning CH, McMahan MR, Chedid M, Keever CA (1987) The major histocompatibility complex of primates. Genetica 73: 53-68

Hermus AR, Sweep CG (1990) Cytokines and the hypothalamic-pituitary-adrenal axis. J Steroid Biochem Mol Biol 37: 867-71

Hibi M, Murakami M, Saito M, Hirano T, Taga T, Kishimoto T (1990) Molecular cloning and expression of an IL-6 signal transducer, gp130. Cell 63: 1149-57

Higuchi M, Ito T, Imai Y, Iwaki T, Hattori M, Kohsaka S, Niho Y, Sakaki Y (1994) Expression of the alpha 2-macroglobulin-encoding gene in rat brain and cultured astrocytes. Gene 141: 155-62

Hill JA (1992) Cytokines considered critical in pregnancy. Am J Reprod Immunol 28: 123-6

Hochereau de Reviers MT, Perreau C (1993) In vitro culture of embryonic disc cells from porcine blastocysts. Reprod Nutr Dev 33: 475-83

Hofig A, Simmen FA, Bazer FW, Simmen RC (1991) Effects of insulin-like growth factor-I on aromatase cytochrome P450 activity and oestradiol biosynthesis in preimplantation porcine conceptuses in vitro. J Endocrinol 130: 245-50

Hopken U, Mohr M, Struber A, Montz H, Burchardi H, Gotze O, Oppermann M (1996) Inhibition of interleukin-6 synthesis in an animal model of septic shock by anti-C5a monoclonal antibodies. Eur J Immunol 26: 1103-9

Hui KM (1989) Re-expression of major histocompatibility complex (MHC) class I molecules on malignant tumor cells and its effect on host-tumor interaction. Bioessays 11: 22-6

Hurwitz A, Payne DW, Packman JN, Andreani CL, Resnick CE, Hernandez ER, Adashi EY, (1991) Cytokine -mediated regulation of ovarian function: interleukin-1 inhibits gonadotropin-induced androgen biosynthesis. Endocrinology 129: 1250-6

Immenschuh S, Song DX, Satoh H, Muller-Eberhard U (1995) The type II hemopexin interleukin-6 response element predominates the transcriptional regulation of the hemopexin acute phase responsiveness Biochem Biophys Res Commun 207: 202-8

Israel A, Le Bail O, Hatat D, Piette J, Kieran M, Logeat F Wallach D, Fellous M, Kourilsky P (1989) TNF stimulates expression of mouse MHC class I genes by inducing an NF kappa B-like enhancer binding activity which displaces constitutive factors. EMBO J 8: 3793-800

Jacobs AL, Sehgal PB, Julian J, Carson DD (1992) Secretion and hormonal regulation of interleukin-6 production by mouse uterine stromal and polarized epithelial cells cultured in vitro. Endocrinology 131: 1037-46

Jaffe L, Jeannotte L, Bikoff EK, Robertson EJ (1990) Analysis of beta 2-microglobulin gene expression in the developing mouse embryo and placenta. J Immunol 145: 3474-82

Jaffe L, Robertson EJ, Bikoff EK (1991) Distinct patterns of expression of MHC class I and beta 2-microglobulin transcripts at early stages of mouse development. J Immunol 147: 2740-9

Jazin EE, Dickerman HW, Henrikson KP (1988) Estradiol stimulates a uterine plasma membrane protease activator. Endocrinology 122: 500-3

Jefcoate CR (1986) "Cytochrome P-450 enzymes in sterol biosynthesis and metabolism". In: Cytochrome P-450, Structure, Mechanism and Biochemistry, edited by P.R. Ortiz de Montellano. Plenum Press, New York and London, pp. 387-428.

Jilka RL, Passeri G, Girasole G, Cooper S, Abrams J, Broxmeyer H Manolagas SC (1995) Estrogen loss upregulates hematopoiesis in the mouse: a mediating role of IL-6. Exp Hematol 23: 500-506

Jones TH, Kennedy RL (1993) Cytokines and hypothalamic-pituitary function. Cytokine 5: 531-8

Jurisicova A, Casper RF, MacLusky NJ, Mills GB, Librach CL (1996) HLA-G expression during preimplantation human embryo development. Proc Natl Acad Sci U S A 93: 161-165

Kauma SW, Matt DW (1995) Coculture cells that express leukemia inhibitory factor (LIF) enhance mouse blastocyst development in vitro. J Assist Reprod Genet 12: 153-6

Kawano M, Hirano T, Matsuda T, Taga T, Horii Y, Iwato K, Asaoku H, Tang B, Tanabe O, Tanaka H, Kuramoto A (1988) Autocrine generation and requirement of BSF-2/IL-6 for human multiple myelomas. Nature 332: 83-5

Kawano MM, Mihara K, Huang N, Tsujimoto T, Kuramoto A (1995) Differentiation of early plasma cells on bone marrow stromal cells requires interleukin-6 for escaping from apoptosis. Blood 85: 487-94

Kellokumpu-Lehtinen P, Talpaz M, Harris D, Van Q, Kurzrock R, Estrov Z (1996) Leukemia-inhibitory factor stimulates breast, kidney and prostate cancer cell proliferation by paracrine and autocrine pathways. Int J Cancer 66: 515-9

Kestler DP, Agarwal S, Cobb J, Goldstein KM, Hall RE (1995) Detection and analysis of an alternatively spliced isoform of interleukin-6 mRNA in peripheral blood mononuclear cells. Blood 86: 4559-67

Keys JL, King GJ (1990) Microscopic examination of porcine conceptus-maternal interface between days 10 and 19 of pregnancy. Am. J. Anat. 188: 221-238

King A, Jokhi PP, Smith SK, Sharkey AM, Loke YW (1995) Screening for cytokine mRNA in human villous and extravillous trophoblasts using the reverse-transcriptase polymerase chain reaction (RT-PCR). Cytokine 7: 364-71

King GJ (1993) Comparative placentation in ungulates. J. Exp. Zool. 266: 588-602

Klein J, O'hUigin C (1993) Composite origin of major histocompatibility complex genes. Curr Opin Genet Dev 3: 923-30

Ko Y, Choi I, Green ML, Simmen FA, Simmen RC (1994) Transient expression of the cytochrome P450 aromatase gene in elongating porcine blastocysts is correlated with uterine insulin-like growth factor levels during peri-implantation development. Mol Reprod Dev 37: 1-11

Kojima K, Kanzaki H, Iwai M, Hatayama H, Fujimoto M, Inoue T, Horie K, Nakayama H, Fujita J, Mori T (1994) Expression of leukemia inhibitory factor in human endometrium and placenta. Biol Reprod 50: 882-7

Kojima K, Kanzaki H, Iwai M, Hatayama H, Fujimoto M, Narukawa S, Higuchi T, Kaneko Y, Mori T, Fujita J (1995) Expression of leukaemia inhibitory factor (LIF) receptor in human placenta: a possible role for LIF in the growth and differentiation of trophoblasts. Hum. Reprod. 10: 1907-1911

Kopf M, Baumann H, Freer G, Freudenberg M, Lamers M, Kishimoto T, Zinkernagel R, Bluethmann H, Kohler G (1994) Impaired immune and acute-phase responses in interleukin-6-deficient mice. Nature 368: 339-42

Kopp E, Ghosh S (1994) Inhibition of NF-kappa B by sodium salicylate and aspirin. Science 265: 956-9

Kordula T, Travis J (1996) The role of Stat and C/EBP transcription factors in the synergistic activation of rat serine protease inhibitor-3 gene by interleukin-6 and dexamethasone. Biochem. J. 313: 1019-27

Koshimizu U, Taga T, Watanabe M, Saito M, Shirayoshi Y, Kishimoto T, Nakatsuji N (1996) Functional requirement of gp130-mediated signaling for growth and survival of mouse primordial germ cells in vitro and derivation of embryonic germ (EG) cells. Development 122: 1235-42

Kover K, Liang L, Andrews GK, Dey SK (1995) Differential expression and regulation of cytokine genes in the mouse uterus. Endocrinology 136: 1666-1673

Kramer KK, Duffy JY, Klemann SW, Bixby JA, Low BG, Pope WF, Roberts RM (1994) Selective cloning of cDNA for secretory proteins of early embryos. Identification of a transiently expressed kunitz domain protein from preimplantation sheep trophoblast. J Biol Chem 269: 7255-61

Kramps JA, Willems LN, Franken C, Dijkman JH (1988) Antileukoprotease, its role in the human lung. Biol Chem Hoppe Seyler 369 Suppl: 83-7

Krams SM, Ascher NL, Martinez OM (1993) New immunologic insights into mechanisms of allograft rejection. Gastroenterol Clin North Am 22: 381-400

Kuida K, Lippke JA, Ku G, Harding MW, Livingston DJ, Su MS, Flavell RA (1995) Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme. Science 267: 2000-3

La Bonnardiere C (1993) Nature and possible functions of interferons secreted by the preimplantation pig blastocyst. J Reprod Fertil Suppl 48: 157-70

Lasfar A, Wietzerbin J, Billard C (1994) Differential regulation of interleukin-6 receptors by interleukin-6 and interferons in multiple myeloma cell lines. Eur J Immunol 24: 124-30

Lavranos TC, Rathjen PD, Seamark RF (1995) Trophic effects of myeloid leukaemia inhibitory factor (LIF) on mouse embryos. J Reprod Fertil 105: 331-8

Layton MJ, Cross BA, Metcalf D, Ward LD, Simpson RJ, Nicola NA (1992) major binding protein for leukemia inhibitory factor in normal mouse serum: identification as a soluble form of the cellular receptor. Proc Natl Acad Sci U S A 89: 8616-20

Lee FD (1992) The role of interleukin-6 in development. Dev Biol 151: 331-8

Li M, Sendtner M, Smith A (1995) Essential function of LIF receptor in motor neurons. Nature 378: 724-7

Li SP, Goldman ND (1996) Regulation of human C-reactive protein gene expression by two synergistic IL-6 responsive elements. Biochemistry 35: 9060-8

Lotem J Sachs L (1992) Hematopoietic cytokines inhibit apoptosis induced by transforming growth factor beta 1 and cancer chemotherapy compounds in myeloid leukemic cells. Blood 80: 1750-7

Lotz M, Moats T, Villiger PM (1992) Leukemia inhibitory factor is expressed in cartilage and synovium and can contribute to the pathogenesis of arthritis J Clin Invest 90(3): 888-96

Lubbert M, Mantovani L, Lindemann A, Mertelsmann R, Herrmann F (1991) Expression of leukemia inhibitory factor is regulated in human mesenchymal cells. Leukemia 5: 361-5

Ludlam WH, Kessler JA (1993) Leukemia inhibitory factor and ciliary neurotrophic factor regulate expression of muscarinic receptors in cultured sympathetic neurons. Dev Biol 155: 497-506

Lutticken C, Wegenka UM, Yuan J, Buschmann J, Schindler C, Ziemiecki A, Harpur AG, Wilks AF, Yasukawa K, Taga T, Kishimoto T, Barbieri G, Pellegrini S, Sendtner M, Heinrich PC, Horn F (1994) Association of transcription factor APRF and protein kinase JAK1 with the interleukin-6 signal transducer gp130. Science 263: 89-92

Machelon V, Emilie D, Lefevre A, Nome F, Durand-Gasselin I, Testart J (1994) Interleukin-6 biosynthesis in human preovulatory follicles: some of its potential roles at ovulation. J Clin Endocrinol Metab 79: 633-42

Mackiewicz A, Laciak M, Gorny A, Baumann H (1993) Leukemia inhibitory factor, interferon gamma and dexamethasone regulate N-glycosylation of alpha 1-protease inhibitor in human hepatoma cells. Eur J Cell Biol 60: 331-6

Maniatis, T, Fritsch, EF, Sambrook, J (1982) Molecular Cloning: A Laboratory Manual. Cold spring Harbor: Cold spring Harbor Laboratory.

Maruyama M, Hay JG, Yoshimura K, Chu CS Crystal RG (1994) Modulation of secretory leukoprotease inhibitor gene expression in human bronchial epithelial cells by phorbol ester. J Clin Invest 94: 368-375

Mathialagan N, Bixby JA, Roberts RM (1992) Expression of interleukin-6 in porcine, ovine, and bovine preimplantation conceptuses. Mol Reprod Dev 32: 324-30

Mathialagan N, Roberts RM (1994) A role for cytokines in early pregnancy Indian J Physiol Pharmacol 38: 153-62

Matsuda T, Yamasaki K, Taga T, Hirano T, Kishimoto T (1989) Current concepts of B cell modulation. Int Rev Immunol 5: 97-109

Matsusaka T, Fujikawa K, Nishio Y, Mukaida N, Matsushima K, Kishimoto T, Akira S (1993) Transcription factors NF-IL6 and NF-kappa B synergistically activate transcription of the inflammatory cytokines, interleukin 6 and interleukin 8. Proc Natl Acad Sci USA 90: 10193-7

May LT, Ghrayeb J, Santhanam U, Tatter SB, Sthoeger Z, Helfgott DC, Chiorazzi N, Grieninger G, Sehgal PB (1988) Synthesis and secretion of multiple forms of beta 2-interferon/B-cell differentiation factor 2/hepatocyte-stimulating factor by human fibroblasts and monocytes. J. Biol. Chem. 263: 7760-7766

Mayer U, Mann K, Timpl R, Murphy G (1993) Sites of nidogen cleavage by proteases involved in tissue homeostasis and remodelling. Eur J Biochem 217: 877-84

McCracken S, Layton JE, Shorter SC, Starkey PM, Barlow DH, Mardon HJ (1996) Expression of granulocyte-colony stimulating factor and its receptor is regulated during the development of the human placenta. J. Endocrinol. 149: 249-258

McMaster MT, Dey SK, Andrews GK (1993) Association of monocytes and neutrophils with early events of blastocyst implantation in mice. J Reprod Fertil 99: 561-9

Metcalf D (1991) The leukemia inhibitory factor (LIF). Int J Cell Cloning 9: 95-108

Meziou W, Chardon P, Flechon JE, Kalil J, Vaiman M (1983) Expression of beta 2-microglobulin on preimplantation pig embryos. J Reprod Immunol 5: 73-80

Miesfeld R L (1989) The structure and function of steroid receptor proteins. Crit Rev Biochem Mol Biol 24: 101-17

Milland J, Loveland BE, McKenzie IF (1993) Isolation of a clone for pig beta 2-microglobulin cDNA. Immunogenetics 38 , 464

Miller WL (1988) Molecular biology of steroid hormone synthesis. Endocrine Reviews 9: 295-318

Minami M, Inoue M, Wei S, Takeda K, Matsumoto M, Kishimoto T, Akira S (1996) STAT3 activation is a critical step in gp130-mediated terminal differentiation and growth arrest of a myeloid cell line. Proc Natl Acad Sci USA 93: 3963-6

Molhuizen HO, Schalkwijk J (1995) Structural, biochemical, and cell biological aspects of the serine proteinase inhibitor SKALP/elafin/ESI. Biol Chem Hoppe Seyler 376: 1-7

Moreau JF, Donaldson DD, Bennett F, Witek-Giannotti J, Clark SC, Wong GG (1988) Leukaemia inhibitory factor is identical to the myeloid growth factor human interleukin for DA cells. Nature 336: 690-2

Morello D, Duprey P, Israel A, Babinet C (1985) Asynchronous regulation of mouse H-2D and beta-2 microglobulin RNA transcripts. Immunogenetics 22: 441-52

Morgan GL, Geisert RD, Zavy MT, Shawley, RV Fazlebas AT (1987) Development of pig blastocysts in an uterine environment advanced by exogenous oestrogen. J Reprod Fertil 80: 125-31

Mossman HW (1987) Vertebral Fetal Membranes. Rutgers University Press, North Brunswick, New Jersey

Motro B, Itin A, Sachs L, Keshet E (1990) Pattern of interleukin 6 gene expression in vivo suggests a role for this cytokine in angiogenesis. Proc Natl Acad Sci U S A 87: 3092-6

Murakami M, Hibi M, Nakagawa N, Nakagawa T, Yasukawa K, Yamanishi K, Taga T, Kishimoto T (1993) IL-6-induced homodimerization of gp130 and associated activation of a tyrosine kinase. Science 260: 1808-10

Murdoch WJ (1995) Immunolocalization of a gonadotropin-releasing hormone receptor site in murine endometrium that mediates apoptosis. Cell Tissue Res 282: 527-529

Murray MK, Malathy PV, Bazer FW, Roberts RM (1989) Structural relationship, biosynthesis, and immunocytochemical localization of uteroferrin-associated basic glycoproteins. J Biol Chem 264: 4143-50

Murray R, Lee F, Chiu CP (1990) The genes for leukemia inhibitory factor and interleukin-6 are expressed in mouse blastocysts prior to the onset of hemopoiesis. Mol Cell Biol 10: 4953-6

Nachtigall M.J, Kliman HJ, Feinberg RF, Olive DL, Engin O, Arici A (1996) The effect of leukemia inhibitory factor (LIF) on trophoblast differentiation: a potential role in human implantation. J Clin Endocrinol Metab 81: 801-6

Navarra P, Tsagarakis S, Faria MS, Rees LH, Besser GM, Grossman AB (1991) Interleukins-1 and -6 stimulate the release of corticotropin-releasing hormone-41 from rat hypothalamus in vitro via the eicosanoid cyclooxygenase pathway. Endocrinology 128: 37-44

Negishi M, Aida K, Yoshioka H (1993) "Sexually dimorphic expression of P-450 genes". In: Cytochrome P-450, 2nd edition, edited by T.Omura, Y.Ishimura and Y. Fujii-Kuriyama. Kodansha, Tokyo,pp.230-42

New MI, Dupont B, Pang S, Pollack MS, Levine LS (1984) "Enzymatic defects of adrenal steroidogenesis". In: Steroid Converting Enzymes and Diseases, Edited by K. Fotherby and S.B. Pal. Walter De Gruyter, Berlin, New York, pp. 1-72

Nicholas NS, Panayi GS (1985) Inhibition of interleukin-2 production by retroplacental sera: a possible mechanism for human fetal allograft survival. Am J Reprod Immunol Microbiol 9: 6-11

Norris JG, Tang LP, Sparacio SM, Benveniste EN (1994) Signal transduction pathways mediating astrocyte IL-6 induction by IL-1 beta and tumor necrosis factor-alpha. VJ Immunol 152: 841-50

Oh SK, Pellegrino MA, Ferrone S, Sevier ED, Reisfeld RA (1975) Soluble HL-A antigens in serum. I. Isolation and purification. Eur J Immunol 5: 161-6

Orava M, Voutilainen R, Vihko R (1989) Interferon-gamma inhibits steroidogenesis and accumulation of mRNA of the steroidogenic enzymes P450scc and P450c17 in cultured porcine Leydig cells. Mol Endocrinol 3: 887-94

Oritani K, Kaisho T, Nakajima K, Hirano T (1992) Retinoic acid inhibits interleukin-6-induced macrophage differentiation and apoptosis in a murine hematopoietic cell line, Y6. Blood 80: 2298-305

Paria BC, Huet-Hudson YM, Dey SK (1993) Blastocyst's state of activity determines the "window" of implantation in the receptive mouse uterus. Proc Natl Acad Sci USA 90: 10159-62

Patten, BM (1948) Embryology of the Pig. 3rd edition, McGraw-Hill Book Company, Inc., New York.

Pease S, Braghetta P, Gearing D, Grail D, Williams RL (1990) Isolation of embryonic stem (ES) cells in media supplemented with recombinant leukemia inhibitory factor (LIF). Dev-Biol 141: 344-52

Peters M, Roeb E, Pennica D, Meyer zum Buschenfelde KH, Rose-John S (1995) A new hepatocyte stimulating factor: cardiotrophin-1 (CT-1). FEBS Lett 372: 177-80

Piquet-Pellorce C, Grey L, Mereau A, Heath JK (1994) Are LIF and related cytokines functionally equivalent? Exp Cell Res 213: 340-7

Pitzel L, Jarry H, Wuttke W (1993) Effects and interactions of prostaglandin F2 alpha, oxytocin, and cytokines on steroidogenesis of porcine luteal cells. Endocrinology 132: 751-6

Pope, WF (1994) "Embryonic mortality in swine." In: Embryonic mortality in domestic species, edited by: M.T. Zavy and R.D. Geisert. CRC Press, Boca Raton, Florida, pp. 53-77.

Portier M, Lees D, Caron E, Jourdan M, Boiron JM, Bataille R, Klein B (1992) Up-regulation of interleukin (IL)-6 receptor gene expression in vitro and in vivo in IL-6 deprived myeloma cells. FEBS Lett 302: 35-8

Pottratz ST, Bellido T, Mocharla H, Crabb D, Manolagas SC (1994) 17 beta-Estradiol inhibits expression of human interleukin-6 promoter-reporter constructs by a receptor-dependent mechanism. J Clin Invest 93: 944-50

Prabhala RH, Wira CR (1995) Sex hormone and IL-6 regulation of antigen presentation in the female reproductive tract mucosal tissues. J Immunol 155: 5566-73

Prather RS (1993) Nuclear control of early embryonic development in domestic pigs. J Reprod Fertil Suppl 48: 17-29

Pronk GJ, Ramer K, Amiri P, Williams LT (1996) Requirement of an ICE-like protease for induction of apoptosis and ceramide generation by REAPER. Science 271: 808-810

Puissant C, Houdebine LM (1990) An improvement of the single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. BioTechnicques 8: 148-9

Pusateri AP, Rothschild MF, Warner CM, Ford SP (1990) Changes in morphology, cell number, cell size and cellular estrogen content of individual littermate pig conceptuses on days 9 to 13 of gestation. J Anim Sci 68: 3727-35

Ramsay AJ, Husband AJ, Ramshaw IA, Bao S, Matthaei KI, Koehler G, Kopf M (1994) The role of interleukin-6 in mucosal lgA antibody responses in vivo. Science 264: 561-3

Ramsoondar J, Christopherson RJ, Guilbert LJ, Wegmann TG (1993) A porcine trophoblast cell line that secretes growth factors which stimulate porcine macrophages. Biol Reprod 49: 681-94

Raqib R, Ljungdahl A, Lindberg AA, Wretlind B, Andersson U, Andersson J (1996) Dissociation between cytokine mRNA expression and protein production in shigellosis. Eur J Immunol 26: 1130-8

Rathjen PD, Toth S, Willis A, Heath JK, Smith AG (1990) Differentiation inhibiting activity is produced in matrix-associated and diffusible forms that are generated by alternate promoter usage. Cell 62: 1105-14

Ray A, Prefontaine KE, Ray P (1994) Down-modulation of interleukin-6 gene expression by 17 beta-estradiol in the absence of high affinity DNA binding by the estrogen receptor. J Biol Chem 269: 12940-6

Ray DW, Ren SG, Melmed S (1996) Leukemia inhibitory factor (LIF) stimulates proopiomelanocortin (POMC) expression in a corticotroph cell line. Role of STAT pathway. J Clin Invest 97: 1852-9

Reddy NR, Wilkie BN, Mallard BA (1996) Construction of an internal control to quantitate multiple porcine cytokine mRNAs by RT-PCR. BioTechniques 21: 868-870

Reed KL, Badinga L, Davis DL, Chung TE, Simmen RC (1996a) Porcine endometrial glandular epithelial cells in vitro: transcriptional activities of the pregnancy-associated genes encoding antileukoproteinase and uteroferrin. Biol Reprod 55: 469-77

Reed KL, Blaeser L, Dantzer V, Green ML, Simmen RC (1996b) Conceptus modulation of antileukopreteinase (ALP) and uteroferrin (UF) gene expression in peri-implantation porcine endometrium. Biol Reprod 54 (Supl 1): 466

Reed MJ, Topping L; Coldham NG, Purohit A, Ghilchik MW, James VH (1993) Control of aromatase activity in breast cancer cells: the role of cytokines and growth factors. J Steroid Biochem Mol Biol 44: 589-96

Richards CD, Shoyab M, Brown TJ, Gauldie J (1993) Selective regulation of metalloproteinase inhibitor (TIMP-1) by oncostatin M in fibroblasts in culture. J Immunol 150: 5596-603

Richards CD, Saklatvala J (1991) Molecular cloning and sequence of porcine interleukin 6 cDNA and expression of mRNA in synovial fibroblasts in vitro. Cytokine 3: 269-76

Rifas L, Kenney JS, Marcelli M, Pacifici R, Cheng SL, Dawson LL, Avioli LV (1995) Production of interleukin-6 in human osteoblasts and human bone marrow stromal cells: evidence that induction by interleukin-1 and tumor necrosis factor-alpha is not regulated by ovarian steroids. Endocrinology. 136: 4056-67

Rivier C, Vale W (1990) Cytokines act within the brain to inhibit luteinizing hormone secretion and ovulation in the rat. Endocrinology 127: 849-56

Roberts RM, Baumbach GA, Saunders PT, Raub TJ, Renegar RH, Bazer FW (1986) Possible function of carbohydrate on glycoproteins secreted by the pig uterus during pregnancy. Mol Cell Biochem 72: 67-79

Roberts RM, Murray MK, Burke MG, Ketcham CM, Bazer FW (1987) Hormonal control and function of secretory proteins. Adv Exp Med Biol 230: 137-50

Roberts RM, Xie S, Trout WE (1993) Embryo-uterine interactions in pigs during week 2 of pregnancy. J Reprod Fertil Suppl 48: 171-86

Robinson RC, Grey LM, Staunton D, Vankelecom H, Vernallis AB, Moreau JF, Stuart DI, Heath JK, Jones EY (1994) The crystal structure and biological function of leukemia inhibitory factor: implications for receptor binding. Cell 77: 1101-16

Roncero C, Fabregat I, Benito M (1995) Regulation of gene expression by interleukin-6 in fetal rat hepatocyte primary cultures: role of epidermal growth factor and dexamethasone. Hepatology 22: 1769-75

Rose TM, Bruce AG (1991) Oncostatin M is a member of a cytokine family that includes leukemia-inhibitory factor, granulocyte colony-stimulating factor, and interleukin 6. Proc Natl Acad Sci U S A 88: 8641-5

Rose TM, Weiford DM, Gunderson NL, Bruce AG (1994) Oncostatin M (OSM) inhibits the differentiation of pluripotent embryonic stem cells in vitro. Cytokine 6: 48-54

Rosenthal A, Wright S, Cedar H, Flavell R, Grosveld F (1984) Regulated expression of an introduced MHC H-2K bm1 gene in murine embryonal carcinoma cells. Nature 310: 415-8

Rozen F, Russo C, Banville D, Zingg HH (1995) Structure, characterization, and expression of the rat oxytocin receptor gene. Proc Natl Acad Sci U S A 92: 200-204

Sallenave JM, Shulmann J, Crossley J, Jordana M, Gauldie J (1994) Regulation of secretory leukocyte proteinase inhibitor (SLPI) and elastase-specific inhibitor (ESI/elafin) in human airway epithelial cells by cytokines and neutrophilic enzymes. Am J Respir Cell Mol Biol 11: 733-41

Samad F, Bergtrom G, Amrani DL (1994) Regulation of plasminogen activation by interleukin-6 in human lung fibroblasts. Biochim Biophys Acta 1221: 307-14

Samuel, CA (1971) The development of pig trophoblast in ectopic sites. J Reprod Fertil 27: 494-5

Sanceau J, Wijdenes J, Revel M, Wietzerbin J (1991) IL-6 and IL-6 receptor modulation by IFN-gamma and tumor necrosis factor-alpha in human monocytic cell line (THP-1). Priming effect of IFN-gamma. J Immunol 147: 2630-7

Santhanam U, Ray A, Sehgal PB (1991) Repression of the interleukin 6 gene promoter by p53 and the retinoblastoma susceptibility gene product. Proc Natl Acad Sci USA 88: 7605-9

Sappino AP, Huarte J, Belin D, Vassalli JD (1989) Plasminogen activators in tissue remodeling and invasion: mRNA localization in mouse ovaries and implanting embryos. J Cell Biol 109: 2471-9

SAS (1988) SAS/STAT User's Guide.6.03 Edition. Cary, NC: Statistical Analysis System Institute, Inc.

Sasaki H, Hirabayashi Y, Ishibashi T, Inoue T, Matsuda M, Kai S, Ikuta K, Yokoyama K, Yokota T, Maruyama Y, Matsuyama S (1995) Effects of erythropoietin, IL-3, IL-6 and LIF on a murine megakaryoblastic cell line: growth enhancement and expression of receptor mRNAs. Leuk Res 19: 95-102

Sato T, Ito A, Mori Y (1990) Interleukin 6 enhances the production of tissue inhibitor of metalloproteinases (TIMP) but not that of matrix metalloproteinases by human fibroblasts. Biochem Biophys Res Commun 170: 824-9

Satoh T, Kobayashi K, Yamashita S, Kikuchi M, Sendai Y, Hoshi H (1994) Tissue inhibitor of metalloproteinases (TIMP-1) produced by granulosa and oviduct cells enhances in vitro development of bovine embryo. Biol Reprod 50: 835-44

Savatier P, Lapillonne H, van Grunsven LA, Rudkin BB, Samarut J (1996) Withdrawal of differentiation inhibitory activity/leukemia inhibitory factor up-regulates D-type cyclins and cyclin-dependent kinase inhibitors in mouse embryonic stem cells. Oncogene 12: 309-22

Schalkwijk J, de Roo C, de Jongh GJ (1991) Skin-derived antileukoproteinase (SKALP), an elastase inhibitor from human keratinocytes. Purification and biochemical properties. Biochim Biophys Acta 1096: 148-54

Schoester M, Heinrich PC, Graeve L (1994) Regulation of interleukin-6 receptor expression by interleukin-6 in human monocytes--a re-examination. FEBS Lett 345: 131-4

Schooltink H, Schmitz Van de Leur H, Heinrich PC, Rose John S (1992) Up-regulation of the interleukin-6-signal transducing protein (gp130) by interleukin-6 and dexamethasone in HepG2 cells. FEBS Lett 297: 263-5

Schumann RR, Kirschning CJ, Unbehaun A, Aberle HP, Knope HP, Lamping N, Ulevitch RJ, Herrmann F (1996) The lipopolysaccharide-binding protein is a secretory class 1 acute-phase protein whose gene is transcriptionally activated by APRF/STAT/3 and other cytokine-inducible nuclear proteins. Mol Cell Biol 16: 3490-503

Schwabe M, Zhao J, Kung HF (1994) Differential expression and ligand-induced modulation of the human interleukin-6 receptor on interleukin-6-responsive cells. J Biol Chem 269: 7201-9

Shen MM, Leder P (1992) Leukemia inhibitory factor is expressed by the preimplantation uterus and selectively blocks primitive ectoderm formation in vitro. Proc Natl Acad Sci USA 89: 8240-4

Shields DC, Harmon DL, Nunez F, Whitehead AS (1995) The evolution of haematopoietic cytokine/receptor complexes. Cytokine 7: 679-88

Simar-Blanchet AE, Paul C, Mercier L, Le Cam A. (1996) Regulation of expression of the rat serine protease inhibitor 2.3 gene by glucocorticoids and interleukin-6. A complex and unusual interplay between positive and negative cis-acting elements. Eur. J. Biochem. 236: 638-648

Simmen RC, Badinga L, Michel FJ (1993) Chromosomal organization of the gene encoding porcine antileukoproteinase and functional analysis of the promoter region in endometrial and placental cells. Mol Cell Endocrinol 97: 101-8

Simmen RC, Baumbach GA, Roberts RM (1988) Molecular cloning and temporal expression during pregnancy of the messenger ribonucleic acid encoding uteroferrin, a progesterone-induced uterine secretory protein. Mol Endocrinol 2: 253-262

Simmen RCM, Green ML, Simmen FA (1995) "The IGF system in periimplantation uterus and embryonic development." In Dey SK (ed.): Molecular and Cellular Aspects of Periimplantation, , Springer-Verlag Press, New York, Inc.

Simmen RC, Michel FJ, Fliss AE, Smith LC, Fliss MF (1992) Ontogeny, immunocytochemical localization, and biochemical properties of the pregnancy- associated uterine elastase / cathepsin-G protease inhibitor, antileukoproteinase (ALP): monospecific antibodies to a synthetic peptide recognize native ALP. Endocrinology 130: 1957-65

Simmen RC, Simmen FA, Bazer FW (1991) Regulation of synthesis of uterine secretory proteins: evidence for differential induction of porcine uteroferrin and antileukoproteinase gene expression. Biol Reprod 44: 191-200

Simmen RC, Simmen FA, Hofig A, Farmer SJ, Bazer FW (1990) Hormonal regulation of insulin-like growth factor gene expression in pig uterus. Endocrinology 127: 2166-74

Singer DS, Camerini-Otero RD, Satz ML, Osborne B, Sachs D, Rudikoff S (1982) Characterization of a porcine genomic clone encoding a major histocompatibility antigen: expression in mouse L cells. Proc Natl Acad Sci U S A 79: 1403-1407

Sinha S, Watorek W, Karr S, Giles J, Bode W, Travis J (1987) Primary structure of human neutrophil elastase. Proc Natl Acad Sci U S A 84: 2228-32

Snyers L, De Wit L, Content J (1990) Glucocorticoid up-regulation of high-affinity interleukin 6 receptors on human epithelial cells. Proc Natl Acad Sci U S A 87: 2838-42

Snyers L; Content J (1992) Enhancement of IL-6 receptor beta chain (gp130) expression by IL-6, IL-1 and TNF in human epithelial cells. Biochem Biophys Res Commun 185: 902-8

Spangelo BL, MacLeod RM, Isakson PC (1990) Production of interleukin-6 by anterior pituitary cells in vitro. Endocrinology 126: 582-6

Stack MS, Moser TL, Pizzo SV (1992) Binding of human plasminogen to basement-membrane (type IV) collagen. Biochem J 284 (Pt 1): 103-8

Stahl J, Gearing DP, Willson TA, Brown MA, King JA, Gough NM (1990) Structural organization of the genes for murine and human leukemia inhibitory factor. Evolutionary conservation of coding and non-coding regions. J Biol Chem 265: 8833-41

Stahl N, Boulton TG, Farruggella T, Ip NY, Davis S, Witthuhn BA, Quelle FW, Silvennoinen O, Barbieri G, Pellegrini S, Ihle JN, Yancopoulos GD (1994) Association and activation of JAK-TYK kinases by CNTF-LIF-OSM-IL-6 beta receptor components. Science 263: 92-5

Steffens GJ, Heinzel-Wieland R, Saunders D, Wolf B, Rudolphus A. (1993) Oxidation resistant muteins of antileukoproteinase as potential therapeutic agents. Agents Actions Suppl 42: 111-21

Stein B, Yang MX (1995) Repression of the interleukin-6 promoter by estrogen receptor is mediated by NF-kappa B and C/EBP beta. Mol Cell Biol 15: 4971-9

Stephanou A, Handwerger S (1994) Interleukin-6 stimulates placental lactogen expression by human trophoblast cells. Endocrinology 135: 719-723

Stephanou A, Myatt L, Eis AL, Sarlis N, Jikihara H, Handwerger S (1995) Ontogeny of the expression and regulation of interleukin-6 (IL-6) and IL-1 mRNAs by human trophoblast cells during differentiation in vitro. J Endocrinol 147: 487-96

Stewart CL (1994) The role of leukemia inhibitory factor (LIF) and other cytokines in regulating implantation in mammals Ann NY Acad Sci 734: 157-65

Stewart CL, Kaspar P, Brunet LJ, Bhatt H, Gadi I, Kontgen F, Abbondanzo SJ (1992) Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. Nature 359: 76-9.

Strickland S, Reich E, Sherman MI (1976) Plasminogen activator in early embryogenesis: enzyme production by trophoblast and parietal endoderm. Cell 9: 231-40

Sui X., Tsuji K, Tajima S, Tanaka R, Muraoka K, Ebihara Y, Ikebuchi K, Yasukawa K, Taga T, Kishimoto T, Nakahata T (1996) Erythropoietin-independent erythrocyte production: signals through gp130 and c-kit dramatically promote erythropoiesis from human CD34+ cells. J Exp Med 183: 837-45

Sweep F, Rijnkels C, Hermus A (1991) Activation of the hypothalamus-pituitary-adrenal axis by cytokines. Acta Endocrinol Copenh, 125 Suppl 1: 84-91

Syed V, Gerard N, Kaipia A, Bardin CW, Parvinen M, Jegou B (1993) Identification, ontogeny, and regulation of an interleukin-6-like factor in the rat seminiferous tubule. Endocrinology 132: 293-9

Symes AJ, Rajan P, Corpus L, Fink JS (1995) C/EBP-related sites in addition to a STAT site are necessary for ciliary neurotrophic factor-leukemia inhibitory factor-dependent transcriptional activation by the vasoactive intestinal peptide cytokine response element. J Biol Chem 270: 8068-75

Tabibzadeh SS, Santhanam U, Sehgal PB, May LT (1989) Cytokine-induced production of IFN-beta 2/IL-6 by freshly explanted human endometrial stromal cells. Modulation by estradiol-17 beta. J Immunol 142: 3134-9

Tabibzadeh S, Kong QF, Babaknia A, May LT (1995) Progressive rise in the expression of interleukin-6 in human endometrium during menstrual cycle is initiated during the implantation window. Hum. Reprod. 10: 2793-2799

Taga T, Hibi M, Hirata Y, Yamasaki K, Yasukawa K, Matsuda T, Hirano T, Kishimoto T (1989) Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. Cell 58: 573-81

Talhouk RS, Chin JR, Unemori EN, Werb Z, Bissell MJ (1991) Proteinases of the mammary gland: developmental regulation in vivo and vectorial secretion in culture. Development 112: 439-49

Tanabe O, Akira S, Kamiya T, Wong GG, Hirano T, Kishimoto T (1988) Genomic structure of the murine IL-6 gene: High degree conservation of potential regulatory sequences between mouse and human. J Immunol 141: 3875-81

Tarleton RL, Koller BH, Latour A, Postan M (1992) Susceptibility of beta 2-microglobulin-deficient mice to Trypanosoma cruzi infection. Nature 356: 338-40

Thatcher WW, Meyer MD, Danet-Desnoyers G (1995) Maternal recognition of pregnancy. J Reprod Fertil Suppl 49: 15-28

Thompson RC, Ohlsson K (1986) Isolation, properties and complete amino acid sequence of human secretory leukocyte protease inhibitor, a potent inhibitor of leukocyte elastase. Proc Natl Acad Sci USA 83: 6692-6

Tess MW, Bennet GL, Dickerson GE (1983) Simulation of genetic changes in life cycle efficiency of pork production. II. Effects of components on efficiency. J Anim Sci 56: 354-68

Tomanek M, Kopecny V, Kanka J (1989) Genome reactivation in developing early pig embryos: an ultrastructural and autoradiographic analysis. Anat Embryol (Berl) 180: 309-16

Tomida M, Yamamoto-Yamaguchi Y, Hozumi M (1993) Pregnancy associated increase in mRNA for soluble D-factor/LIF receptor in mouse liver. FEBS Lett 334: 193-7

Tominaga T, Fukata J, Naito Y, Usui T, Murakami N, Fukushima M, Nakai Y, Hirai Y, Imura H (1991) Prostaglandin-dependent in vitro stimulation of adrenocortical steroidogenesis by interleukins. Endocrinology 128: 526-31

Townsend AR (1987) Recognition of influenza virus proteins by cytotoxic T lymphocytes. Immunol Res 6: 80-100

Trautwein C, Caelles C, van der Geer P, Hunter T, Karin M, Chojkier M (1993) Transactivation by NF-IL6/LAP is enhanced by phosphorylation of its activation domain. Nature 364: 544-7

Truss M, Beato M (1993) Steroid hormone receptors: interaction with deoxyribonucleic acid and transcription factors. Endocr Rev 14: 459-79

Vandenbergh-DJ; Mori-N; Anderson-DJ (1991) Co-expression of multiple neurotransmitter enzyme genes in normal and immortalized sympathoadrenal progenitor cells. Dev Biol 148: 10-22

Vercelli D, Jabara HH, Geha RS (1989) Regulation of human IgE synthesis. Int Rev Immunol 5: 111-5

Vogelmeier C, Hubbard RC, Fells GA, Schnebli HP, Thompson RC, Fritz H, Crystal RG (1991) Anti-neutrophil elastase defense of the normal human respiratory epithelial surface provided by the secretory leukoprotease inhibitor. J Clin Invest 87: 482-8

Wang J, Chen S (1992) Identification of a promoter and a silencer at the 3'end of the first intron of the human aromatase gene. Mol Endocrinol 6: 1479-88

Wang Y, Fuller GM (1994) Phosphorylation and internalization of gp130 occur after IL-6 activation of Jak2 kinase in hepatocytes. Mol Biol Cell 5: 819-28

Waterhouse P, Denhardt DT, Khokha R (1993) Temporal expression of tissue inhibitors of metalloproteinases in mouse reproductive tissues during gestation. Mol Reprod Dev 35: 219-26

Werb Z, Alexander CM, Adler RR (1992) Expression and function of matrix metalloproteinases in development. Matrix Suppl 1: 337-43

Wijdenes J, Heinrich PC, Muller-Newen G, Roche C, Gu ZJ, Clement C, Klein B (1995) Interleukin-6 signal transducer gp130 has specific binding sites for different cytokines as determined by antagonistic and agonistic anti-gp130 monoclonal antibodies. Eur J Immunol 25: 3474-3481

Williams RL, Hilton DJ, Pease S, Willson TA, Stewart CL, Gearing DP, Wagner EF, Metcalf D, Nicola NA, Gough NM (1988) Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature 336: 684-7

Willson TA, Metcalf D, Gough NM (1992) Cross-species comparison of the sequence of the leukaemia inhibitory factor gene and its protein. Eur J Biochem 204: 21-30

Woessner JF (1979) Separation of collagenase and a metal-dependent endopeptidase of rat uterus that hydrolyzes a heptapeptide related to collagen. Biochim Biophys Acta 571: 313-20

Yamaguchi M, Michishita M, Hirayoshi K, Yasukawa K, Okuma M, Nagata K (1992) Down-regulation of interleukin 6 receptors of mouse myelomonocytic leukemic cells by leukemia inhibitory factor. J Biol Chem 267: 22035-42

Yamaguchi M, Taga T, Kishimoto T, Miyake A (1995) Cytokines that use gp130 as a signal transducer stimulate mouse placental lactogen-I (mPL-I) but inhibit mPL-II production in vitro. Biol Reprod 53: 399-406

Yamasaki K, Taga T, Hirata Y, Yawata H, Kawanishi Y, Seed B, Taniguchi T, Hirano T, Kishimoto T (1988) Cloning and expression of the human interleukin-6 (BSF-2/IFN beta 2) receptor. Science 241: 825-8

Yang CH, Shi W, Basu L, Murti A, Constantinescu SN, Blatt L, Croze EMullersman JE, Pfeffer LM (1996) Direct association of STAT3 with the IFNAR-1 chain of the human type I interferon receptor. J Biol Chem 271: 8057-61

Yang ZM; Le SP; Chen DB; Harper MJ (1994) Temporal and spatial expression of leukemia inhibitory factor in rabbit uterus during early pregnancy. Mol Reprod Dev 38: 148-52

Yasuda K, Fukuoka M, Fujiwara H, Mori T (1992) Effects of interferon on the steroidogenic functions and proliferation of immature porcine granulosa cells in culture. Biol Reprod 47: 931-6

Yasukawa K, Hirano T, Watanabe Y, Muratani K, Matsuda T, Nakai S, Kishimoto T (1987) Structure and expression of human B cell stimulatory factor-2 (BSF-2/IL-6) gene. EMBO J 6: 2939-45

Ying QL, Kemme M, Simon SR (1994) Functions of the N-terminal domain of secretory leukoprotease inhibitor. Biochemistry 33: 5445-50

Yoshida K, Chambers I, Nichols J, Smith A, Saito M, Yasukawa K, Shoyab M, Taga T, Kishimoto T (1994) Maintenance of the pluripotential phenotype of embryonic stem cells through direct activation of gp130 signalling pathways. Mech Dev 45: 163-71

Zhang Z, Paria BC, Davis DL (1991) Pig endometrial cells in primary culture: morphology, secretion of prostaglandins and proteins, and effects of pregnancy. J Anim Sci 69: 3005-15

Zhao Y, Nichols JE, Bulun SE, Mendelson CR, Simpson ER (1995) Aromatase P450 gene expression in human adipose tissue. Role of a JAK/STAT pathway in regulation of the adipose-specific promoter. J Biol Chem 270: 16449-57

Zhong Z, Wen Z, Darnell JE (1994) Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. Science 264: 95-8

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Tomislav Modrić was born in Zagreb, Croatia, on August 21, 1961. He graduated with the degree in veterinary medicine in June 1987, from the University of Zagreb. During the next three years he held three different positions, at the Department for Biology and Pathology of Fish and Bees at the University of Zagreb (1987-1988), at a large animal practice in Velika Kladuša, Bosnia and Herzegovina (1988-1989), and in the Croatian Center for Reproduction of Domestic Animals (1989-1990). In 1990 he came to the USA, where he obtained a Master of Science degree in biology from the University of Texas at El Paso in July 1992. He started his Ph.D. program in the Department of Physiological Sciences at the University of Florida in August 1992. He joined Dr. Frank A. Simmen's laboratory in July 1994 to complete the requirements for the degree of Doctor of Philosophy in January, 1997. He will pursue postdoctoral research in the laboratory of Dr. Liam J. Murphy in the Department of Physiology at the University of Manitoba, Canada, beginning April, 1997.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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